Des complexes protéiques thermo-induits fonctionnels : quelles interactions établissent-ils dans un gel acide laitier ?

Marion Morand

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Table des matières

Liste des abréviations

α-la : α-lactalbumine
ANS : acide 8-anilino-1-naphtalène sulfonique
CLSM : confocal laser scanning microscope
D_h : diamètre hydrodynamique
DLS : *dynamique light scattering* (diffusion dynamique de la lumière)
DMA : 2-diméthylaminoéthanothiol hydrochloride
DTNB : acide 5,5'-dithio-bis-2-nitrobenzoïque
DTT: dithiothreitol
EDC : N-3-diméthylaminopropyl-N-éthylcarbodiimide hydrochloride
G' : module élastique
G'' : module visqueux
GDL : glucono-δ-lactone
HPLC : *reverse Phase-High Performance Liquid Chromatography* (chromatographie liquide en phase inverse)
IRTF : spectroscopie infrarouge à transformée de fourrier
LC/MS : *liquid chromatography*–mass *spectrometry* (chromatography liquide-spectroscopie de masse)
MALLS : *multi-angle laser light scattering* (diffusion statique ou multiangles de la lumière)
mb : mobilité électrophorétique
MUF *milk ultrafiltration permeate* (ultrafiltrat de lait)
M_w : *molecular weight* (masse moléculaire)
NEM : N-éthylmaléimide
NMC : *native micellar casein*
OPA : ortho-phthaldialdehyde
PCA : *principal component analysis* (analyse en composantes principales)
pI : point isoélectrique ou pH de charge résiduelle nulle
PPCN : caséine micellaire native
PSH : *protein surface hydrophobicity index* (hydrophobicité de surface des protéines)
R_g : rayon de giration
RITC : rhodamine B isothiocyanate
S : résidu cystéine
SDS : dodecyl sulfate de sodium
SEC : *size exclusion chromatography* (chromatographie d'exclusion stérique)
SH : sulfhydryle libre
SH/SS : échange thiol-disulfure
SS : pont disulfure
STLO : science et technologie du lait et de l'œuf
tan δ : *loss tangent* (tangente de perte ou rapport G'/G")
TCA : acide trichloroacétique
TEM: *transmission electron microscopy* (microscopie électronique en transmission)
WPI : *whey protein isolate* (isolat de protéines solubles)
β-lg : β-lactoglobuline

Chapitre I.

Introduction

I.1. Contexte socio-économique du projet

La transformation du lait en yaourt et autres produits fermentés a utilisé environ 7% de la collecte française de lait mais a généré 2.5 milliards d'euros de chiffre d'affaire en 2009, soit près de 13% du chiffre d'affaire des industries laitières nationales (Centre national interprofessionnel de l'économie laitière, 2010). Le secteur des yaourts et produits fermentés est très dynamique avec une évolution de +9% (en volume produit) entre 2002 et 2008 (FranceAgriMer, 2010). Il bénéficie d'une forte consommation de la part des français (0.7 yaourt/jour/habitant). Les 3 transformateurs leaders sur le marché des yaourt et desserts lactés, en France, sont Danone, Lactalis et Andros mais ils sont peu représentés dans la région Bretagne qui comptabilise seulement 6% de la production nationale (FranceAgriMer, 2010).

Les yaourts sont des produits fragiles et évolutifs, les défauts de qualités majeures et récurrents de ces produits sont l'exsudation de sérum et des problèmes de texture et de consistance des gels. Pour limiter ces défauts, les industriels ont recours à des traitements thermiques énergivores et au poudrage du lait, c'est-à-dire l'opération qui consiste à augmenter l'extrait sec du lait par l'ajout de poudres laitières. Les réductions énergétiques et l'utilisation de coproduits de l'industrie laitière valorisés en ingrédients fonctionnels, dans le but de maitriser la qualité des produits et de développer l'innovation, constituent des enjeux pour toute la filière. En tant que grand producteur de poudres laitières avec 109 000 tonnes produites en 2009 (France AgriMer, 2010), la région Bretagne investit dans ce sens dans la recherche fondamentale avec pour objectif la compréhension des mécanismes de formation des gels acides. La région Bretagne a ainsi financé le projet de recherche présenté dans ce document par une bourse d'allocation de recherche doctorale (ARED) numéro 42 98.

I.2. Questions de recherche

Il est maintenant admis que le traitement thermique du lait à 85-95°C pendant plusieurs minutes entraîne l'apparition de complexes thermo-induits protéiques résultant de la dénaturation des protéines sériques. Il a été clairement établi que ces complexes sont impliqués dans l'amélioration des propriétés de gélification acide du lait : ils augmentent significativement le pH de gélification, la rétention d'eau, la fermeté et l'homogénéité des gels acides de type yaourt. Depuis les années 80, de nombreuses études ont permis d'avancer sur la compréhension de la formation des complexes thermo-induits et sur les interactions mises en jeu entre ces complexes et avec la micelle de caséines au cours du traitement thermique du lait (Figure 1). D'après des études récentes, quelques auteurs (Alexander & Dalgleish, 2005; Donato et al., 2007a; Guyomarc'h et al., 2009a) soutiennent l'hypothèse que les complexes thermo-induits interagissent avec la surface de la micelle de caséine au cours de l'acidification et la fonctionnaliseraient, en améliorant les propriétés de gélification acide. Cependant, on ne sait encore que peu de choses sur les mécanismes de formation des gels acides laitiers (Figure 1).

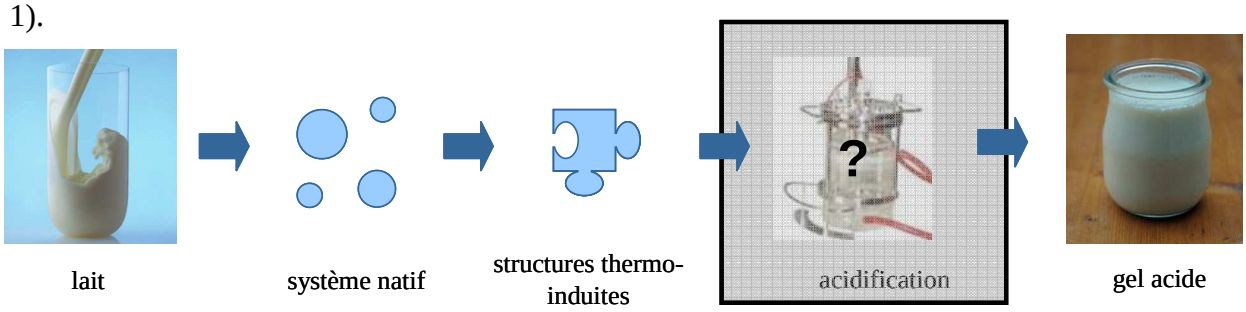

Figure 1 : Synthèse de l'état des connaissances sur les conséquences du traitement thermique sur le lait écrémé et sur son aptitude à la gélification acide

Dans le but de faire progresser la formulation et la prescription des ingrédients fonctionnels utilisés en gélification acide, la communauté scientifique se focalise maintenant sur les questions suivantes :

- Quelle est la nature des interactions survenant entre les complexes thermo-induits et les micelles de caséines lors de la formation des gels ?

- Comment ces interactions conduisent à la déstabilisation du lait et la construction du réseau protéique ?

Liste des communications réalisées au cours du projet

Articles dans des journaux internationaux à comité de lecture

Morand M, Guyomarc'h F et Famelart M-H, 2011, «How to tailor heat-induced whey protein/κ-casein complexes, as a means to investigate the acid gelation of milk - a review», *Dairy Science and Technology,* 91, pp 97-126, DOI: 10.1007/s13594-011-0013-x **[article 1]**

Morand M, Pezennec S, Guyomarc'h F et Famelart M-H, 2011, «On how κ-casein affects the interactions between the heat-induced whey protein/κ-casein complexes and the casein micelles during the acid gelation of skim milk», *International Dairy Journal,* 21, pp 670-678, DOI: 10.1016/j.idairyj.2011.01.012 **[article 2]**

Morand M, Legland D, Guyomarc'h F et Famelart M-H, «Changing the isoelectric point of the heat-induced whey protein complexes affects the acid gelation of skim milk», accepté dans *International Dairy Journal* **[article 3]**

Morand M, Dekkari A, Guyomarc'h F et Famelart M-H, « Increasing the hydrophobicity of the heat-induced whey protein complexes improves the acid gelation of skim milk », en préparation **[article 4]**

Articles dans des journaux nationaux

Morand M, Guyomarc'h F et Famelart M-H, 2010 « Ingénierie des nanostructures protéiques et propriétés rhéologiques des gels acides de lait », *Groupe français de rhéologie 2010*

Famelart M-H, Guyomarc'h F, Morand M et Novales B, 2011, « Agrégation protéique et propriétés gélifiantes et moussantes des protéines laitières – quoi de neuf sur le plan des connaissances ? », *Innovations Agronomiques*

Communication dans des congrès

Conférences dans des congrès internationaux

Morand M, Guyomarc'h F et Famelart M-H, 2010, "Designing the heat-induced whey protein complexes: a key to engineer the interactions in acid milk gels", Word Dairy Summit, Auckland, New Zealand

Morand M, Guyomarc'h F et Famelart M-H, 2010, « On how heat-induced whey protein/k-casein complexes affect interactions during the acid gelation of milk », IDF symposium « Microstructure », Tromsø, Norway

Conférences dans des congrès nationaux

Morand M, Dekkari A, Guyomarc'h F et Famelart M-H, 2011, « Ingénierie des nanoparticules protéiques et propriétés rhéologiques des gels acides laitiers », Rencontres de Biologie Physique du Grand Ouest, Rennes

Famelart M-H, Guyomarc'h F, Morand M et Novales B, 2011, « Agrégation protéique et propriétés gélifiantes et moussantes des protéines laitières », Carrefour de l'innovation agronomique, Protéines laitières, Rennes

Morand M, Guyomarc'h F et Famelart M-H, 2010, « Ingénierie des nanostructures protéiques et propriétés rhéologiques des gels acides de lait » Groupe français de rhéologie, Rhéologie des matériaux nanostructurés, Lyon

Posters

Morand M, Guyomarc'h F et Famelart M-H, 2010, « Changing the isoelectric point of the heat-induced whey protein complexes affect the acid gelation of skim milk », Biopolymères, Le Croisic, France

Autres communications

Morand M, 2011, « Les complexes protéiques thermo-induits : vecteurs de nouvelles propriétés fonctionnelles des micelles de caséines », posters des doctorants de l'UMR STLO, Rennes, France

Barbe F, Bouzerzour K, Morand M et Van Audenhaege M, 2010, court métrage : " La vie d'une protéine de lait", festival science en court, Nicomaque Rennes, France

Distinctions

Obtention d'une bourse de la Fédération Internationale de Laiterie de France (FIL France) pour la participation au congrès Word Dairy Summit 2010, Auckland, New Zealand.

Obtention d'une bourse DCIColl de la Région Bretagne, pour la participation au congrès Word Dairy Summit 2010, Auckland, New Zealand.

Chapitre II.

Etude bibliographique

II.1. Les protéines du lait

Le lait contient deux catégories de protéines : les protéines sériques et les caséines. Les protéines sériques sont définies comme étant l'ensemble des protéines solubles à pH 4.6 par opposition aux caséines qui précipitent à ce pH. La proportion de ces protéines dans le lait ainsi que quelque unes de leurs propriétés sont résumées dans le Tableau 1**.**

Tableau 1 : Composition du lait en protéines (Cayot & Lorient, 1998; Rasic & Kurmann, 1978; 2005) et quelques propriétés de ces protéines (Walstra et al., 2006).

		Composition dans le lait ($g\ L^{-1}$)	Masse moléculaire (kDa)	Nombre de résidus cystéines (dont sulfhydriles libres) (par mol)	Point isoélectrique (pI)
Caséines (~29 $g\ L^{-1}$)	Caséine-α_{S1}	11.1	23.6	0	4.5
	Caséine-β	10.4	24.0	0	4.8
	Caséine-κ	3.7	19.0	2 (0)	5.6
	Caséine-α_{S2}	2.9	25.2	2 (0)	5.0
Protéines Sériques (~7 $g\ L^{-1}$)	β-lactoglobuline	2.5-4.5	18.3	5 (1)	5.2
	α-lactalbumine	1.2-1.7	14.2	8 (0)	~4.3
	Albumine de sérum bovin	0.5	66.3	35 (1)	4.8

II.1.1. Les protéines sériques

De nature et de fonction diverses, les protéines sériques sont aussi très sensibles aux traitements thermiques (voir §II.4). Les principales protéines sériques sont la β-lactoglobuline (β-lg) et l'α-lactalbumine (α-la).

La β-lg est la protéine la plus abondante du lactosérum (~50%). La β-lg est une protéine globulaire et de masse moléculaire de 18.3 kDa, dont la structure est très compacte par la présence de nombreux feuillets β. Elle présente également 2 ponts disulfures, entre le résidu cystéine en position 66 (Cys_{66}) et Cys_{160} et entre Cys_{106} et Cys_{119}, et un groupement sulfhydrile

libre (Cys_{121}), ce dernier étant proche du cœur hydrophobe et donc non accessible sur la forme native de la protéine.

L'α-la est également une protéine globulaire et sa masse moléculaire est de 14.2 kDa. Elle contient 4 ponts disulfures : Cys_6-Cys_{120}, Cys_{28}-Cys_{111}, Cys_{61}-Cys_{77} et Cys_{73}-Cys_{91}.

D'autres protéines sériques mineures sont également présentes dans la phase soluble du lait, notamment l'albumine de sérum bovin, les immunoglobulines et la lactoferrine.

II.1.2. Les caséines et la micelle de caséines

Les caséines représentent 80% (p/p) des protéines du lait (Cayot & Lorient, 1998) et elles sont insolubles à pH 4.6. Il existe plusieurs types de caséines : les caséines-α_{s1}, α_{s2}, β et κ, dont les concentrations dans le lait écrémé sont données dans le Tableau 1. Les caséines sont des chaînes d'acides aminés à faible organisation secondaire. Elles s'assemblent ensemble sous forme de particules colloïdales, ou micelles de caséines, de diamètre compris entre 30 et 600 nm, avec une moyenne de 180 nm (Cayot & Lorient, 1998).

Selon les différents modèles de la structure de la micelle de caséines décrits actuellement, modèle « cœur enveloppe » (Waugh et al., 1970), à « structure ouverte » (Holt et al., 1989; Holt, 1992; Horne, 1998) ou encore à submicelles (Morr, 1967; Ono & Obata, 1989; Schmidt, 1982), les caséines-κ seraient plutôt situées à la surface de la micelle, liées par leur partie N-terminale aux parties hydrophobes des autres caséines et présentent une partie chargée, hydrophile à la surface de la micelle formant ainsi une couche ou des patches de « chevelure » d'une dizaine de nanomètres d'épaisseur (Holt & Dalgleish, 1986; Walstra, 1990). Dans l'édifice micellaire, les caséines sont maintenues les unes aux autres grâce aux interactions hydrophobes et à la présence de phosphate de calcium colloïdal (PCC). En dépit de leur taille et de l'instabilité naturelle des caséines individuelles en milieu aqueux et salin, les micelles de caséines sont remarquablement résistantes à la concentration, au séchage ou au traitement thermique. Cette stabilité s'explique d'une part par l'exclusion stérique engendrée par la couche chevelue de caséine-κ, qui interdit le contact proche d'une autre micelle. D'autre part, les micelles de caséines sont très hydratées et leur masse volumique de 1.06 g mL^{-1} n'excède celle du perméat de lait que de 4 mg mL^{-1} (Mahaut et al., 2000). En agitation Brownienne permanente, les micelles restent ainsi facilement en suspension. Enfin, les micelles de caséines

possèdent une charge résiduelle nette de l'ordre de -10 à -20 mV (Horne, 2006; Walstra, 1990), et exercent une répulsion électrostatique les unes sur les autres. Ces répulsions stériques et électrostatiques sont compensées par des forces attractives de longue portée, dites de Van der Waals. La résultante de ces forces produit le système colloïdal équilibré qu'est le lait. Dans certains procédés comme la coagulation fromagère ou la gélification acide, la déstabilisation du lait est recherchée et sera obtenue par des moyens qui affectent cet équilibre. L'acidification du lait est notre procédé d'intérêt.

II.2. La gélification acide du lait

Les mécanismes physico-chimiques impliqués dans la formation des gels acides ont été présentés dans une récente revue de Lee & Lucey (2010) :

- de pH 6.7 à 6.0

Les charges négatives de la micelle de caséines diminuent, ce qui entraîne une diminution des répulsions électrostatiques. Une petite quantité du PCC est solubilisée à pH> 6.0 mais la taille des micelles de caséines est inchangée.

- de pH 6.0 à 5.0

Les charges négatives de la micelle de caséines diminuent fortement et la couche de « chevelure » de la caséine-κ s'effondre et s'amincit, par un phénomène de collapse. Il en résulte une diminution des répulsions électrostatiques, de la stabilisation stérique, et de la couche d'hydratation qui sont toutes les trois responsables de la stabilité de la micelle de caséine dans le lait. A l'approche de pH 5.0, le taux de solubilisation du PCC augmente, tandis que le calcium lié directement aux phosphoserines est solubilisé, ce qui affaiblit la structure interne des micelles de caséines. On observe alors une dissociation partielle des caséines. La micelle se contracte.

- de pH ≤ 5.0

Lorsque le pH devient proche du point isoélectrique de la micelle de caséines (pH 4.6), les répulsions électrostatiques entre les molécules de caséine diminuent. D'autre part, des

attractions hydrophobes et électrostatiques (+/-) s'établissent entre les particules de caséine. Le processus d'acidification résulte en la formation d'un réseau tridimensionnel constitué de clusters et de chaînes de caséines.

Dans les gels acides alimentaires, l'acidification est classiquement réalisée par des bactéries lactiques (*Lactobacillus bulgaricus* et *Streptococcus thermophilus*) qui métabolisent le lactose et sécrètent de l'acide lactique. Pour obtenir une acidification correcte par les ferments, la température d'incubation du lait doit être d'environ 40-45°C. Dans les études modèles, la glucono-δ-lactone (GDL) est communément utilisée pour s'affranchir des variabilités biologiques des ferments. La GDL s'hydrolyse et libère de l'acide gluconique. Les cinétiques d'acidification d'un lait acidifié avec de la GDL ou avec des ferments cependant sont très différentes (Figure 2) (Lucey et al., 1998a). La GDL s'hydrolyse rapidement tandis que les ferments acidifient plus lentement le lait. La quantité de GDL détermine le pH final tandis que les ferments ne peuvent acidifier en deçà de pH ~4.0, où leur croissance est inhibée. Lucey et al. (1998a) ont observé des différences de propriété de gélification d'un lait acidifié avec de la GDL ou avec des ferments à la même valeur de pH final. Les auteurs ont observé un module élastique et une expulsion du sérum plus importants pour les gels acidifiés par GDL que pour ceux produits avec ferments.

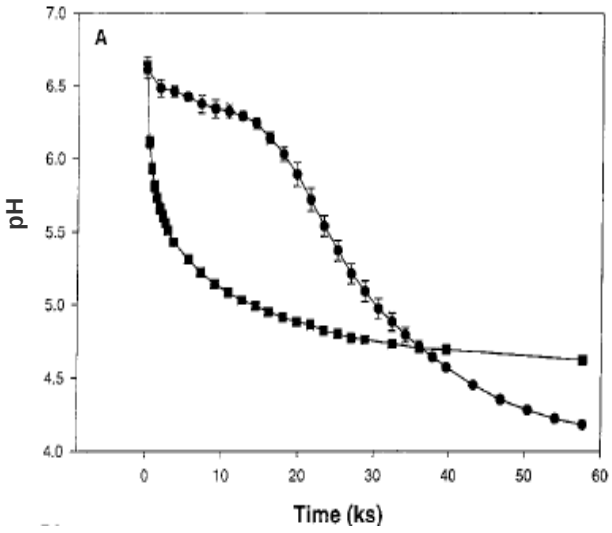

Figure 2 : Evolution du pH au cours de l'acidification d'un lait avec 1.3% (p/p) de la glucono-δ-lactone (GDL) (■) ou 2% (p/p) d'une culture bactérienne (●) (Lucey et al., 1998a).

Quel que soit le mode d'acidification, l'augmentation des températures d'incubation des laits (>40°C vs <40°C) conduit à une accélération de la vitesse d'acidification, une diminution du module élastique à pH 4.6 et une augmentation de l'exsudation de sérum des gels acides (Lee

& Lucey, 2010). Les gels formés à hautes températures (>40°C) sont plus faibles et présentent de plus larges pores que ceux formés à faibles températures (<40°C). En modulant ce facteur, il est possible d'infléchir la cinétique d'acidification de la GDL de façon à ce qu'elle soit comparable à la phase exponentielle de l'acidification lactique (Renan et al., 2008). Malgré ces limites, la GDL reste donc un moyen scientifique intéressant pour acidifier de façon standard des laits plus ou moins modifiés, ce que ne peuvent pas faire des bactéries dont la croissance dépendra de la composition de leur substrat.

II.3. Effets du chauffage du lait sur les gels acides

En industrie, pour des raisons sanitaires et technologiques, le lait destiné à la fabrication de gels acides est chauffé à 85-95°C pendant une dizaine de minutes dans le but d'éliminer les bactéries et les enzymes contaminantes et indésirables. En effet, cela éliminera la flore endogène du lait, les ferments lactiques ensemencés se développeront plus facilement et le gel acide présentera des propriétés améliorées. Typiquement, un lait cru ou pasteurisé gélifie à l'approche du point isoélectrique des micelles (pH ~4.9) et le gel obtenu à pH 4.5 atteint un module élastique G' de l'ordre de la dizaine de Pa. Un lait chauffé 90°C/10 min gélifie à une valeur de pH plus élevée, généralement entre 5.1 et 5.6 ; son module élastique à pH 4.5 atteint plusieurs centaines de Pa et sa capacité de rétention d'eau est améliorée par rapport à un lait cru (Figure 3).

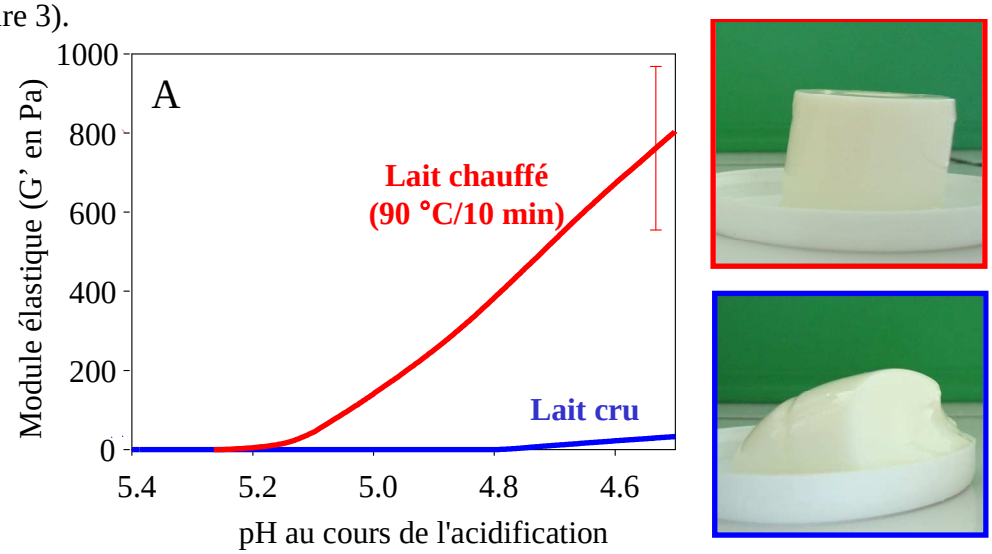

Figure 3 : Evolution du module élastique (G') au cours de l'acidification d'un lait cru et d'un lait chauffé (à gauche). Photographies d'un gel acide de lait cru (en bas) et d'un lait chauffé (en haut) d'après Jemin et al. (2008).

II.4. Effets du chauffage du lait sur les protéines du lait

Les micelles sont peu sensibles à la dénaturation thermique, mais une partie des caséines, en particulier la caséine-κ, peut se dissocier de la micelle et se retrouver dans la phase soluble d'un lait chauffé entre 70 et 100°C (Walstra et al., 2006).

Les protéines sériques sont quant à elles dénaturées à des températures supérieures à 60°C. Des modifications conformationnelles des protéines sériques provoquent l'exposition de leurs groupements hydrophobes et/ou thiols. Les protéines sériques peuvent s'agréger entre elles et avec les caséines, essentiellement avec la caséine-κ et α_{S2}, par des échanges thiol/disulfides (SH/SS) et par des liaisons non covalentes de type interactions hydrophobes, ioniques ou de Van der walls, (Guyomarc'h et al., 2003b; Jang & Swaisgood, 1990; Noh et al., 1989; Singh & Creamer, 1991; Smits & van Brouwershaven, 1980) dans des proportions très variables selon les conditions expérimentales (pH, concentration en sels, température) (Galani & Apenten, 1999). Les entités produites sont appelées complexes thermo-induits. Le terme « agrégats » est également courant dans la littérature, mais peut porter confusion avec le produit de la déstabilisation acide du lait. Les complexes peuvent être formés à la surface des micelles (complexes micellaires) ou être formés en phase soluble (complexes solubles) selon leur localisation dans le lait (Figure 5). La β-lg a beaucoup été étudiée comme modèle des protéines sériques, afin de comprendre les mécanismes de dénaturation/agrégation dans le lait et les systèmes laitiers. Plusieurs scénarios sont envisagés dans la littérature. Ils ont été regroupés dans la revue de Donato et Guyomarc'h (2009) dont une adaptation est présentée en Figure 5. La première alternative relevée par les auteurs repose sur la nécessité de formation ou non de complexes primaires de β-lg ou de β-lg/α-la dénaturées pour la formation de complexes solubles et micellaires avec la caséine-κ. La deuxième question alternative porte sur le rôle de la micelle de caséines sur la distribution des complexes entre la phase micellaire et soluble du lait : la dissociation de la caséine-κ a-t-elle lieu avant ou après son interaction avec les protéines sériques (ou avec les complexes primaires)? Donato & Guyomarc'h (2009) soulignent que la dominance d'un mécanisme sur l'autre, par des variations du milieu par exemple, peut éventuellement influencer les interactions mises en jeu dans les complexes lors

de leur formation, et influencer la spécificité des propriétés fonctionnelles des complexes solubles ou micellaires.

Jean et al. (2006) ont isolé et caractérisé les complexes solubles d'un lait chauffé à 90°C/10 min. Ces complexes solubles, illustrés sur la Figure 4, sont constitués majoritairement de protéines sériques et de caséine-κ liées principalement par des ponts disulfures. Leur diamètre hydrodynamique est d'environ 70 nm, leur point isoélectrique (pI ou pH de charge nulle) est d'environ 4.5 et leur surface est plus hydrophobe que celle de la micelle.

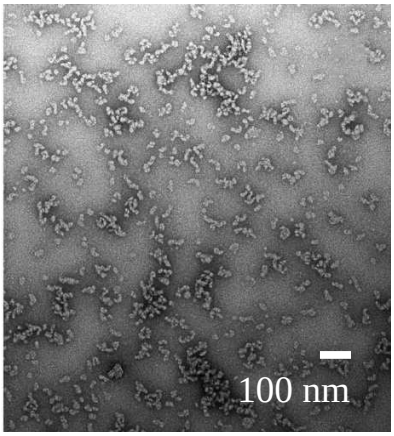

Figure 4 : Image en microscopie électronique à transmission de complexes solubles thermo-induits d'un lait chauffé à 90°C/10 min (Jean et al., 2006)

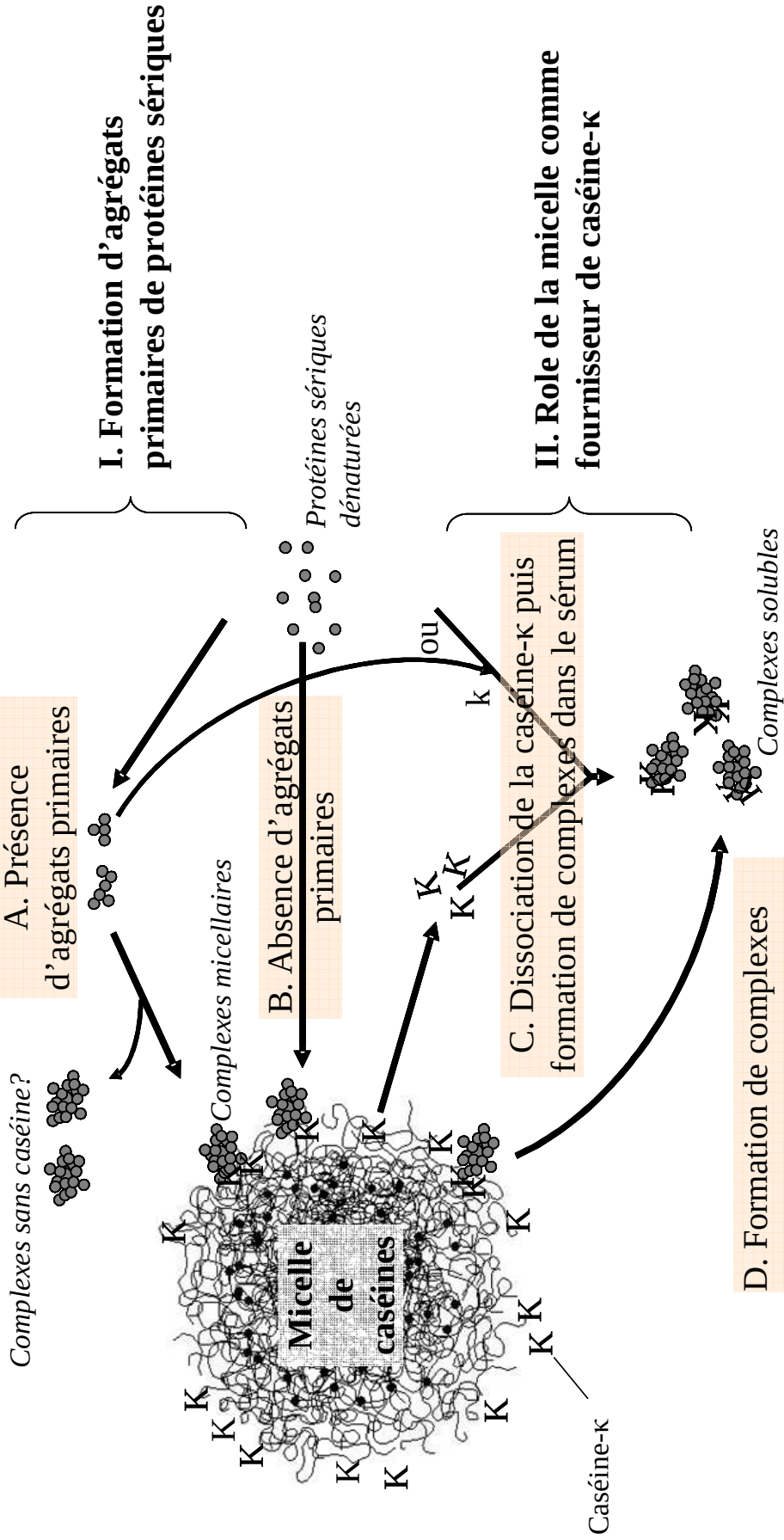

Figure 5 : Schéma des différents mécanismes de formation des complexes solubles et micellaires dans le lait au cours du traitement thermique, d'après Famelart et al. (2011) et Donato et al. (2009). Deux premières alternatives sont possibles (I), qui reposent sur l'existence ou pas d'agrégats primaires constitués de protéines sériques uniquement (respectivement les voies A et B). Le mode d'interaction entre la caséine-κ et les protéines sériques est également en débat (II), avec une solubilisation préalable de caséine-κ, puis des interactions formées en phase sérique (voie C) ou une interaction ayant lieu sur la micelle et une solubilisation ultérieure des complexes (voie D)

II.5. Rôle des complexes thermo-induits dans la gélification acide du lait chauffé

La formation d'un gel acide précoce et ferme suite au chauffage du lait est bien corrélée avec la dénaturation des protéines sériques (Dannenberg & Kessler, 1988a; Dannenberg & Kessler, 1988b; Lucey et al., 1997a; Parnell-Clunies et al., 1986a) et la formation de complexes thermo-induits (Kalab et al., 1976; Lucey et al., 1998b; Vasbinder et al., 2003a). Quel que soit leur localisation (complexes solubles ou micellaires), ces complexes participent à la formation du réseau protéique de gels acides de lait, micelles et complexes étant co-localisés dans le gel (Guyomarc'h et al., 2009a; Vasbinder et al., 2003a). De plus, quelle que soit la localisation des complexes thermo-induits, plus il y a de complexes et plus les gels acides sont fermes et précoces (Donato et al., 2007a; Guyomarc'h et al., 2007b). Des complexes solubles, obtenus par le chauffage de protéines sériques en solution et ajoutés à un lait cru sans protéines sériques, permettent également la formation d'un gel ferme et précoce (Guyomarc'h, 2002; O'Kennedy & Kelly, 2000; Schorsch et al., 2001). Ainsi, de nombreux auteurs soutiennent l'hypothèse que les complexes solubles interagissent avec la micelle au point de gel et même avant la gélification (Alexander & Dalgleish, 2005; Donato et al., 2007a; Guyomarc'h et al., 2009a). La micelle serait le matériel de construction du gel acide et les complexes, attachés à leur surface apporteraient de nouvelles fonctionnalités à la micelle, et plus précisément, augmenteraient leur aptitude à intéragir. Il a ainsi été proposé que la fonctionnalisation de la surface des micelles de caséines par les complexes thermo-induits permettrait aux micelles :

- d'augmenter les attractions entre les particules, en augmentant leur hydrophobie (Guyomarc'h et al., 2007b),
- d'augmenter la rigidité des connexions, en introduisant des ponts disulfures dans le réseau protéique et éventuellement en en créant de nouveaux pendant la gélification (Vasbinder et al., 2003a),
- d'augmenter le nombre d'interactions entre les micelles, pontées par les complexes (Guyomarc'h et al., 2003a; Lakemond & Van Vliet, 2008; Lucey et al., 1998b),

- d'augmenter l'anisotropie des interactions et ainsi former un gel acide composé de brins linéaires et de larges pores.

Pour résumer, la Figure 6 illustre le principe de fonctionnalisation de la micelle de caséines par les complexes thermo-induits. Au cours de l'acidification, les complexes thermo-induits fonctionnaliseraient la surface de la micelle de caséines et lui apporteraient de nouvelles propriétés d'interaction. La gélification du lait chauffé apparaitrait donc vers le pI des micelles fonctionnalisées, c'est-à-dire vers le pI des complexes présents à leur surface.

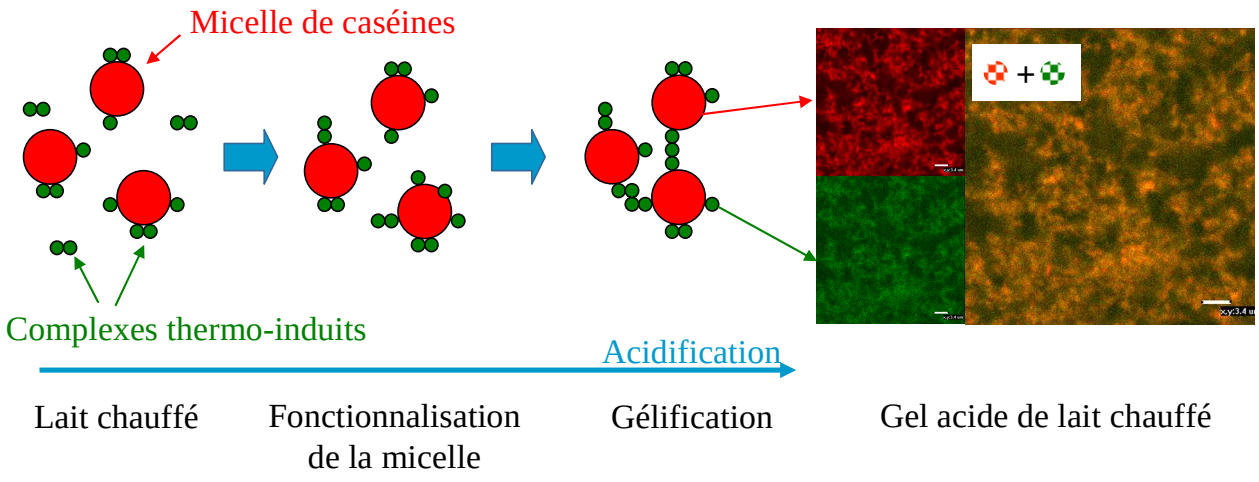

Figure 6 : Schéma du principe de fonctionnalisation de la micelle par les complexes thermo-induits au cours de l'acidification du lait. Images en microscopie confocale d'un gel acide dont les micelles et les complexes ont été marquée séparément avec des sondes différentes représentées en rouge et en vert, respectivement (Guyomarc'h et al., 2009a). Le gel acide, mélange micelle et complexes (photo de droite), apparaît en orange, somme de la couleur verte et rouge. Les 2 sondes sont donc co-localisées dans le gel. Cette colocalisation est vérifiée tout au long de l'acidification (ici pH 4.5).

II.6. Etude des interactions protéiques au cours de la gélification du lait chauffé

II.6.1. Caractérisation du type de liaisons et des interactions constitutives des gels acides

Certains auteurs ont étudié le type d'interactions constitutives des gels acides en modifiant les paramètres de l'acidification (température, présence de sels…) et en caractérisant les propriétés mécaniques des gels formés (Lucey et al., 1997b; Lucey et al., 1997c; Lucey et al., 1998b; Roefs & Van Vliet, 1990; Surel & Famelart, 2003). Une autre approche a également été

utilisée qui consiste à rompre spécifiquement les liaisons protéiques présentes dans le gel constitué et à caractériser les protéines ou particules libérées par le traitement (Alting et al., 2004; Lefebvre-Cases et al., 1998; Lucey et al., 1999; Vasbinder et al., 2003a). Ainsi, les interactions électrostatiques, hydrophobes et hydrogène et les liaisons disulfures ont été mises en évidence comme constitutives des gels acides de lait. Les conclusions de ces travaux sont proposées dans les paragraphes suivants. Notons que ce type d'approche n'a pas permis d'évaluer l'importance d'un certain nombre d'interactions (constructives ou de stabilisation) pouvant intervenir sur la construction des gels acides (les interactions de Van der waals, les ponts hydrogène, les gènes stériques, la solvatation des particules…). De plus, en étudiant uniquement le gel acide final, ces travaux n'ont pas apporté d'information sur le mécanisme de la gélification acide, en particulier le rôle de ces interactions sur la déstabilisation du lait ou sur la façon dont ces intéractions peuvent se succéder au cours de l'acidification.

II.6.1.1. Interactions électrostatiques

Les interactions électrostatiques sont essentiellement dues aux groupements ionisables des protéines. Les groupements carboxyliques terminaux et les résidus aspartique et glutamique ainsi que les groupements phosphate de la caséine sont susceptibles d'être porteurs de charges négatives, tandis que les groupements amines terminaux et les résidus guadinine et histidine peuvent être porteurs de charges positives. L'état de charge de ces groupements, qui dépend de leur pK, du pH et de la force ionique du milieu, engendre des forces attractives ou répulsives. Pour chaque protéine, il existe un pH particulier, pour lequel le bilan de charge est nul, c'est le point isoélectrique (pI).

Les interactions électrostatiques répulsives participent à la stabilité des protéines en suspension. Lorsque les charges des protéines sont réduites par une acidification les particules peuvent se rapprocher et s'agréger si les forces attractives le permettent. En effet, la gélification des micelles de caséines ou des protéines sériques dénaturées a lieu vers leur pI, à 4.8 ou ~5.4, respectivement.

Les interactions électrostatiques attractives semblent également participer à la construction des gels acides de lait. Roefs & van Vliet (1990) expliquent la diminution du module élastique

des gels acides avec l'ajout de NaCl par la réduction des attractions électrostatiques, le sel écrantant les charges des protéines.

II.6.1.2. Interactions hydrophobes

Les interactions hydrophobes résultent du caractère apolaire de certains groupements qui ont tendance à se regrouper afin d'éviter le contact avec la phase aqueuse. La chaleur favorise le rapprochement des groupements hydrophobes tandis que le froid ou la présence de détergents comme le dodecyl sulfate de sodium (SDS) ou de solvants organiques réduit les interactions hydrophobes.

L'implication des interactions hydrophobes dans la construction des gels acides de lait est fortement suspectée (Dalgleish & Law, 1988; Van Vliet et al., 1989). En effet, un lait acidifié à froid (<4°C) ne gélifie pas. Il ne gélifie que lorsque la température augmente. En revanche, sur une gamme de 20 à 50°C, la température d'incubation du lait pendant l'acidification semble avoir un effet contradictoire. Lorsque la température d'incubation est basse, le module élastique des gels est plus fort qu'un gel incubé à haute température (Lucey et al., 1997b; Lucey et al., 1997c; Lucey et al., 1998b; Roefs & Van Vliet, 1990). Ceci peut être dû à un effet du à la vitesse d'acidification, mais les auteurs expliquent qu'à haute température, les fortes interactions hydrophobes provoquent des réarrangements entre les particules au cours de la gélification qui sont défavorables à la construction d'un gel ferme.

Lefebvre-Cases et al. (1998) et Surel et al. (2003) ont montré la forte implication des interactions hydrophobes dans la construction des gels acides de lait. L'ajout de SDS dans les gels, utilisé pour rompre les liaisons hydrophobes en augmentant les répulsions électrostatiques intramoléculaires, provoque la dissociation quasi-totale des protéines du gel.

II.6.1.3. Ponts disulfures

Les ponts disulfures (SS) sont des liaisons covalentes établies entre 2 résidus cystéine. Ils peuvent se former par oxydation de 2 sulfhydryles libres (SH) ou par échange thiol-disulfure (SH/SS).

Il est bien connu que les complexes thermo-induits sont formés par des échanges SH/SS lors du chauffage. Le blocage des SH avant le chauffage par le N-éthylmaléimide (NEM) conduit à

une diminution importante du module élastique (G') des gels acide de lait (Lucey et al., 1998b). Mais cette diminution du G' lorsque le NEM est ajouté au lait après chauffage est faible selon Vasbinder (2003b) (20 % de réduction du G' du gel) et nulle selon Bouchebbah et al. (2010). Selon Vasbinder (2003b), la diminution du G' en présence de NEM n'apparaît qu'après 10 h de gélification. Les interactions SS n'interviendraient pas dans les interactions initialement formées entre les particules du lait chauffé, mais éventuellement plutôt au cours de la réorganisation du gel.

Il semble donc que le G' des gels acides soit plus lié à la composition en ponts SS intraparticulaires constitutifs des complexes que ceux interparticulaires pouvant se former au cours de l'acidification. Il est à noter que l'approche qui consiste à rompre les interactions SS formées au cours de l'acidification par ajout d'un agent réducteur, afin de montrer leur implication ne peut pas être appliquée ici, puisque l'agent réducteur conduirait à réduire tous les ponts SS présents dans le gel acide, ceux formés au cœur des complexes thermo-induits et ceux formés au cours de l'acidification.

II.6.1.4. Interactions de nature hydrogène

Les interactions hydrogène peuvent se former entre un atome électronégatif possédant au moins un doublet électronique (O, N, S) et un atome d'hydrogène lui-même lié à un atome électronégatif. Ces liaisons intra ou intermoléculaires jouent un rôle déterminant dans la stabilisation des structures. Les protéines peuvent également former de nombreux ponts hydrogène avec les molécules d'eau du solvant, ce qui contribue à la structure et à la solubilité des protéines. Ces liaisons sont détruites par la chaleur, l'urée, le chlorure de guanidine et sont renforcées par le froid.

Comme précédemment (II.6.1.2), Lefebvre-Cases et al. (1998) ont suggéré l'implication d'interactions hydrogène dans la stabilisation des gels acides par l'ajout d'urée qui rompt les liaisons hydrogène intramoléculaires. Notons que l'urée peut également rompre les liaisons hydrophobes.

II.6.2. L'ingénierie des complexes protéines sériques/caséine-κ comme moyen d'étude de la gélification acide

II.6.2.1. Introduction

Pour aller plus loin dans la compréhension des interactions et des liaisons constitutives des gels acides laitiers, et en particulier, pour appréhender des notions mécanistiques, la stratégie originale du projet de thèse consiste à utiliser les complexes thermo-induits aux propriétés physico-chimiques modifiées, comme des vecteurs de nouvelles fonctionnalités des micelles de caséines. Dans cette perspective, il est nécessaire d'inventorier les propriétés physico-chimiques des complexes susceptibles de modifier la gélification acide des micelles de caséines et d'évaluer les moyens physiques, chimiques et biologiques pour les obtenir. Cette revue bibliographique a fait l'objet d'une publication (Morand et al., 2011a) proposée ci-dessous.

II.6.2.2. Article 1

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REVIEW PAPER

How to tailor heat-induced whey protein/κ-casein complexes as a means to investigate the acid gelation of milk—a review

Marion Morand · Fanny Guyomarc'h · Marie-Hélène Famelart

Abstract - The heat treatment of milk greatly improves the acid gelation of milk and is therefore largely applied in yoghurt manufacture. During the heat treatment, soluble and micelle-bound whey protein/κ-casein complexes are produced in milk. The complexes and their physico-chemical properties have been held responsible for the early gelation point, the increased final firmness and for the serum retention capacity of the acid gels made of heated milk. They are suspected to bring new functionalities to the casein micelles and to help the formation of interactions when building the gel network. In order to investigate the type of interactions that the complexes can affect throughout the acid gelation of milk, an original strategy would be to control the physico-chemical properties of the whey protein/κ-casein soluble complexes and to use them as vectors to modify the possible interactions in the milk. In that perspective, the different physico-chemical properties of the whey protein/κ-casein soluble complexes that are thought to significantly affect the acid gelation behaviour of the casein micelles are listed. Then, the physical, chemical and biological means that could possibly be applied to the formation of complexes in order to modulate each of the targeted property are reviewed and evaluated. In order to open a large choice for future investigation, these methods were found in a larger literature resource than milk, including other protein systems like model whey protein solutions or non-dairy globular protein systems. The food-compatible character of some of these means is indicated, for their potential technological interest.

Résumé - Le traitement thermique du lait améliore considérablement la gélification acide du lait et est très largement appliqué pour la fabrication des yaourts. Pendant le traitement thermique, des complexes solubles et micellaires composés de protéines sériques et de caséine κ sont produits dans le lait. Les complexes et leurs propriétés physico-chimiques sont présumés responsables de la gélification acide précoce, de l'augmentation de fermeté et de la rétention d'eau des gels acides obtenus à partir de lait chauffés. Ils sont supposés apporter de nouvelles fonctionnalités à la micelle de caséines et participer ainsi à la formation des interactions lors de la construction du réseau. Dans le but de progresser dans la connaissance des interactions que ces complexes sont susceptibles de modifier dans le gel acide de lait, une stratégie originale consisterait à contrôler les propriétés physico-chimiques des complexes protéines sériques/caséine κ solubles et de les utiliser comme vecteurs pour modifier les interactions dans le lait. Dans cette perspective, le but de cette revue est d'inventorier et d'évaluer les moyens physiques, chimiques et biologiques de moduler les propriétés des complexes protéines sériques/caséine κ susceptibles de modifier la gélification acide des micelles de caséine. Les moyens recensés proviennent de travaux réalisés soit dans le lait, soit dans des systèmes protéiques laitiers, comme les solutions modèles de protéines sériques, ou dans des systèmes protéiques non laitiers. Le caractère alimentaire de certains de ces moyens est souligné, en tant que pistes technologiques.

1. Introduction

Milk proteins, which weight for 33-35 g kg^{-1} of total skim milk, are essential to the formation of dairy gels like cheese or yogurt. Milk proteins commonly fall into the whey proteins, accounting for 7-8 g kg^{-1} and found in globular soluble form; and the caseins, accounting for 26-28 g kg^{-1} and found in colloidal assemblies called the casein micelles (Walstra & Jenness, 1984a). In bovine milk, the two major whey proteins are the β-lactoglobulin (2-4 g kg^{-1}) and α-lactalbumin (~1.5 g kg^{-1}), followed by the immunoglobulins, bovine serum albumin and lactoferrin. The caseins are the α_{s1}-casein, β-casein (9-10 g kg^{-1} each), κ-casein and α_{s2}-casein (2-4 g kg^{-1} each; (Cayot & Lorient, 1998). In all types of dairy gels, the casein micelles are the essential building blocks of the protein network (de Kruif,

1998; Holt & Horne, 1996; Van Vliet et al., 1989). In their native form, the whey proteins barely contribute to gelation, neither through acidification nor renneting. However, it is well-known that denaturation of the whey proteins largely accounts for the effect of heat treatment on the acid gelation of milk. Typically, acidified milk that had been heated at 85-95°C for several minutes starts to gel at a higher pH value than unheated milk and yields an acid gel with increased elastic modulus and higher water retention capacity (Lucey et al., 1997a; Lucey et al., 2000; Van Vliet et al., 2004).

In the literature, different reviews exist that thoroughly describe the heat-induced denaturation and aggregation of the whey proteins, either in model solutions of β-lactoglobulin or of whey protein isolates (de Wit, 2009; Foegeding et al., 2002) or in milk (Donato & Guyomarc'h, 2009). In essence, the whey proteins unfold as soon as the temperature exceeds ~60°C then interact through hydrophobic interactions and thiol/disulphide exchanges to yield heat-induced whey protein complexes. In milk, these complexes also involve the cysteine-containing κ-casein and can be found both in the form of soluble complexes and in the form of micelle-bound complexes (Anema, 1997; Anema & Li, 2000; Donato & Dalgleish, 2006; Guyomarc'h et al., 2003b). However, despite the increased knowledge on the structural and physico-chemical properties of these heat-induced whey protein/κ-casein complexes, little is known of the actual mechanism through which they can affect the onset of gelation and the final mechanical properties of the acid milk gel. In their review, Donato & Guyomarc'h (2009) suggested that the whey protein/κ-casein complexes bring new functionalities to the casein micelles, either through modifying their surface as to increase the attraction-to-repulsion resultant interaction or through acting as spacers that bridge the micelles together and sustain the gel's microstructure. In order to investigate these hypotheses, one original approach would be to design model whey protein/κ-casein complexes having one targeted physico-chemical property largely modulated, then to introduce them to whey protein-free milk as a means to promote (or knock out) one specific type of interaction during acidification. If this modulation induces a correlated change in the acid gelation behaviour of the milk, the corresponding interaction may then be identified as important to the formation of the acid milk gel. Because they are separated from the casein micelles and because the acid gelation functionality of the heat-induced whey protein/κ-casein complexes does not seem to depend on their soluble or

micelle-bound location (Donato et al., 2007a; Guyomarc'h et al., 2009a), the soluble complexes seem good candidates to be used as vectors to modulate interactions in milk.

In this perspective, the present review will first overview, out of recent research (Alting et al., 2002; Alting et al., 2004; Alting et al., 2003; Donato et al., 2007b; Guyomarc'h et al., 2009a; Guyomarc'h et al., 2007b; Guyomarc'h et al., 2009b), the different physico-chemical properties of the soluble whey protein/κ-casein complexes that may be important to either the onset of acid gelation (in a first section) or the strengthening of the acid gel network (in a second section). Then, the various physical, biological or chemical means that could help control each listed property in either section will be reviewed. The review therefore aims at providing a set of methods that may help modify whey protein/κ-casein soluble complexes produced in model systems (Donato et al., 2007b; Guyomarc'h et al., 2009a) or isolated from milk. Should this approach be too restrictive, the review also provides methods to generate derivative whey protein, food protein or food protein/κ-casein heat-induced complexes through introducing controlled changes, e.g. in the protein composition of the heat-induced whey protein/κ-casein complexes. In order to open a large range of opportunities for future investigation, the choice was made to seek these methods not only in studies on milk, but also in studies on milk protein fractions, on isolated milk proteins or on non-dairy globular food proteins. In the latter cases, the applicability of the methods to the whey protein/κ-casein complexes or their derivatives is yet to be evidenced. On the other hand, these literature areas bring new resources to import methods from.

In heated milk, it is well-known that the heat-induced whey protein/κ-casein complexes are parted between the serum and the colloidal phases of milk (Anema, 2007; Guyomarc'h et al., 2003b; Vasbinder & de Kruif, 2003). Because of the chosen strategy to introduce modified soluble whey protein/κ-casein complexes as vectors to modulate interactions in milk, the means to control the serum/micelle partition of the heat-induced complexes in milk are out of the scope of the present review. Information can be found in Donato & Guyomarc'h (2009). For the same reason, the heat-induced changes in the composition and structure of the casein micelles, as well as their possible consequences on acid gelation, are excluded.

2. Properties of the heat-induced whey protein/κ-casein complexes that affect the onset of acid gelation in milk

2.1. Possible roles of the heat-induced whey protein/κ-casein complexes on the acid destabilisation of milk

In the search for an appropriate model to describe their structure and account for their behaviour in a number of processes, the stability of casein micelles in skim milk has long been thoroughly discussed (Dalgleish, 1998; de Kruif, 1998; Holt & Horne, 1996; Horne, 1998; Tuinier & de Kruif, 2002; Walstra, 1990). In essence, destabilisation of the casein micelle may occur through their disintegration or aggregation. On acidification to pH values ~5.2, dissolution of the colloidal calcium phosphate and the increase in hydration of the casein molecules essentially account for loosening of the casein micelles' structure (Banon & Hardy, 1992; Walstra, 1990). Collapse of the outer κ-casein brush as pH decreases to ~5.0, neutralisation of the electrostatic repulsion between negatively charged casein particles as pH approaches pI ~4.8 and the occurrence of dipole-dipole or hydrophobic attractions further yield to gelation (Banon & Hardy, 1992; Tuinier & de Kruif, 2002; Van Vliet et al., 1989). As the casein micelles loosen and rearrange throughout acidification (Gastaldi et al., 1996), the exact nature of the casein "micelles" that eventually form the particulate gel is not known, which sensibly challenges modelling (Horne, 2003). In heated milk, the presence of heat-induced whey protein/κ-casein complexes increases the pH at which gelation onsets from pH ~4.8 to ~5.4 (Graveland-Bikker & Anema, 2003; Guyomarc'h et al., 2003a; Heertje et al., 1985; Lucey et al., 1997a). It is hypothesized that the complexes modify the surface properties of casein micelles as to increase net colloidal attraction, hence decreasing the threshold for acid destabilisation (Donato & Guyomarc'h, 2009).

Among the forces that balance interaction between the casein micelles during acidification of the milk, electrostatic repulsion and the pI value are clearly important (Tuinier & de Kruif, 2002). Since the pI can determine the pH of gelation of heat-induced whey protein complexes (Alting et al., 2002), the pH of gelation of milk may similarly depend on the pI of the heat-induced whey protein/κ-casein complexes. Attractive hydrophobic interaction has also been

emphasized through the effect of temperature (Banon & Hardy, 1992). As they carry significant surface hydrophobicity, it was suggested that the heat-induced whey protein/κ-casein complexes also promoted destabilisation through increasing attraction (Famelart et al., 2004; Guyomarc'h et al., 2007b; Jean et al., 2006). Calculation showed that long-range van der Waals attraction also counterbalances electrostatic repulsion and may account for aggregation below pH 5 (Tuinier & de Kruif, 2002). Despite the above literature, there is to date no general picture of how the heat-induced whey protein/κ-casein complexes contribute to increasing the pH at which acid gelation starts in heated milk, i.e. through which of the above-listed interaction(s) they favour precipitation of the casein micelles. It is however expected that:

- they easily interact with the surface of the casein micelles.
- they reinforce hydrophobic attraction and/or reduce electrostatic repulsion.

2.2. Possible methods to modify the surface charge and apparent pI of the heat-induced whey protein/κ-casein complexes

In milk ultrafiltration permeate, the soluble whey protein/κ-casein complexes isolated from heated skim milk have a pI of ~4.5, a net charge of -18 mV at pH 6.7 and they start precipitating at pH 5.3-5.5 on acidification (Guyomarc'h et al., 2007b; Jean et al., 2006). A first, natural approach to help modify the net charge of the heat-induced whey protein/κ-casein complexes could be to use biodiversity. The major whey protein β-lactoglobulin (β-lg) comes in a range of up to 11 genetic variants, of which the A (ASP_{64}, VAL_{118}) and B forms (GLY_{64}, ALA_{118}) are most represented (Farrell et al., 2004). Although the negative net charge of β-lg A is known to be higher than that of β-lg B, it only slightly affected the pH of acid gelation of an AB skim milk to which up to 1.4 g kg^{-1} of either variant had been added prior to heat treatment (Bikker et al., 2000). In common bovine breeds, κ-casein also comes in two major variants, A (THR_{136}, ASP_{148}) and B (ILE_{136}, ALA_{148}). On the basis of their sequence only, κ-casein is weakly negatively charged and variant B slightly more than A (Cayot & Lorient, 1998). However, the net charge of κ-casein is further increased by the large range of phosphorylated and glycosylated forms (Vreeman et al., 1986), κ-casein B being also more glycosylated than A (Robitaille et al., 1991). However, despite cumulated sources of charge on variant B, and

despite some variant-specific changes in the heat-induced interaction of κ-casein with β-lg (Allmere et al., 1998b; Robitaille & Ayers, 1995) no or barely significant changes could be reported in the acid gelation kinetics of preheated individual milk genotypes (Allmere et al., 1998a). These results probably rule out the biodiversity of β-lg and κ-casein as a means to modulate the net charge of the heat-induced whey protein/κ-casein complexes across a sufficiently large range.

It may be otherwise possible to modulate the net charge and/or the apparent pI of heat-induced whey protein/κ-casein complexes by transferring methods used outside the conditions of milk. Bovetto et al. (2007) and Schmitt et al. (2009) have for instance delivered a method to produce heat-induced nanoparticles of β-lg having similar hydrodynamic diameter (typically 160-230 nm) but opposite net charges of + 25–30 or -30–40 mV, using heat treatment of pure β-lg at pH 4.6 or 5.8, respectively, in deionised water. Since the resulting complexes are partially built on disulphide covalent bonds, some conformational stability, hence residual charge difference, may be expected if the β-lg complexes are later transferred to a near-neutral medium like milk.

Another approach to try to modulate the overall charge and apparent pI of heat-induced whey protein/κ-casein complexes could be to involve cysteine-containing exogenous globular protein into the complexes, with special interest in proteins having pI values away from those of the casein micelles and the heat-induced whey protein/κ-casein complexes, i.e., 4.2-4.5 (Guyomarc'h et al., 2007b). Prior to heat treatment, skim milk could for instance be reinforced in basic proteins like lactoferrin (pI ~8.7) or egg lysozyme (pI ~11) which are both able to aggregate with at least α-lactalbumin (α-la; (Nigen et al., 2009; Takase, 1998)). Soy glycinin (pI ~6.4) and conglycinin (pI ~4.9) could also be helpful to modulate the pI of heat-induced globular protein/κ-casein complexes. Roesch & Corredig (2006; 2005) and Roesch et al. (2004) have for instance shown that the co-heating of pure soy and whey protein isolates in 0.1 M NaCl at pH 7, of soy protein isolate and skim milk, or of soy protein isolate, whey proteins and casein micelles in milk ultrafiltration permeate, produced heat-induced soy/whey or soy/whey/κ-casein soluble complexes that yielded acid gelation profiles with an increased gelation pH value of ~6.0. Egg ovalbumin (pI ~4.8) was also effectively co-heated with whey

protein-depleted skim milk to produce complexes that increased the pH of acid gelation of the modified milk (Famelart et al., 2004; Famelart et al., 2003). However, since β-lg has a higher pI (~5.3) than ovalbumin, charge was obviously not the only driving factor to earlier destabilisation of the milk.

Although it is somewhat less acceptable in food processing, chemical or enzymatic modification of milk proteins may also be considered in order to modify the surface charge of the heat-induced whey protein/κ-casein complexes. Alting et al. (2002) for instance used succinylation of free amino groups to increase the net charge and decrease the pI of heat-induced complexes of either pure β-lg or whey protein isolate produced in water at pH 7.2. They reported a remarkable correlation between the apparent pI of the grafted complexes and their pH of gelation on acidification. They also indicated that it was possible to increase the pI, hence the gelation pH, of heat-induced β-lg or whey protein isolate (WPI) complexes through methylation of the carboxylic groups. However, the results were not shown. Sitohy et al. (1995) also succeeded in decreasing the pI of β-lg through chemical phosphorylation mediated by aliphatic amines.

Non-specific fixation of charged ligands using e.g. anionic surfactants like sodium dodecyl sulphate (SDS) onto the hydrophobic patches of the heat-induced whey protein/κ-casein complexes may also be attempted to help increase their net charge. Risso et al. (2000) for instance reported that the net charge of heated casein micelles resuspended in aqueous Tris-HCl-$CaCl_2$ buffer at pH 6.4 could be increased by the binding of anionic flurorescent probes. Jung et al. (2008) further reported that the double-coating of β-lg heat-induced complexes by SDS in water through successive electrostatic and hydrophobic interactions could eventually reverse their net charge. However, application of the method to whey protein/κ-casein complexes may have consequences on their structure, especially in the case of surfactants, because they are partially built on hydrophobic interactions (Jean et al., 2006). When the complexes are introduced into milk, the ligands may also diffuse and cross-bind to casein micelles, thus rendering the control of colloidal interactions difficult.

Food-grade alternatives exist that may prove less flexible. A first one may be to attach sugars to the ε-amines of the whey protein/κ-casein complexes through Maillard reaction.

Indeed, one can expect to decrease the pI of the complexes through glycation of the amines. Using this approach, the pH of minimum solubility or of isoelectric focusing of pure β-lg can for instance be shifted from ~5.0 to ~4.0 (Chevalier et al., 2001; Corzo-Martinez et al., 2008). It is also conceivable to reduce the pI of β-lg through Maillard conjugation with acidic oligosaccharides (Hattori et al., 2004). To the author's knowledge, no data is yet available on the use of the Maillard reaction to modify the pI of heat-induced whey protein or whey protein/κ-casein complexes. Morgan et al. (1999) albeit showed that heat-induced glycation and polymerization of β-lg could occur concomitantly in solution at 60°C and yield heat-induced complexes that partially remained soluble at pH 4.6.

Second, enzymatic cleavage of the κ-casein's negatively charged glycosyl group using neuraminidase, or of the caseinomacropeptide using chymosin, could also be attempted to modify the net charge of heat-induced whey protein/κ-casein complexes. The method has been shown to increase the pH of acid gelation of the casein micelles in milk (Cases et al., 2003; Gastaldi et al., 1996; Li & Dalgleish, 2006; Niki et al., 2003). Renan et al. (2007), Anema et al. (2007) and Mollé et al. (2006) showed that κ-casein as involved in the heat-induced whey protein/κ-casein complexes of milk could also be cleaved by chymosin. Reduction of the electronegativity of the complexes may then account for the increase in the pH of gelation of heated skim milk after moderate renneting (Guyomarc'h et al., 2007b; Li & Dalgleish, 2006). However, to the authors' knowledge, no demonstration has been given that the pI of the heat-induced whey protein/κ-casein complexes actually increased with renneting nor that the pH of gelation of milk would equally increase if only the heat-induced complexes were renneted.

A wide literature otherwise exists on performing chemical grafting, enzymatic cleavage or ligand addition onto the constitutive protein (generally β-lg or other globular food model protein) prior to their heat-induced aggregation. But, it is then quite likely to affect the denaturation and aggregation processes themselves and hence, to also modify other properties than the charge of the complexes (e.g. Mine (1996); Broersen et al. (2006; 2007) on ovalbumin, Giroux & Britten (2004); Chakraborty et al. (2009); Bouhallab et al. (1999); Chobert et al. (2006) on whey proteins or glycated β-lg and Murphy & Howell (1990)). Modifying the pI of the constituted complex, using the above mentioned techniques, therefore

seems a preferred approach to evaluate the role of electrostatic interactions in the acid gelation of milk.

In conclusion, numerous methods exist that may be applied to the heat-induced whey protein/κ-casein complexes in order to modulate their electrostatic properties. In essence, the complexes preferably precipitate across a pH range that is centred at their pI value but also depends on how their absolute net charge increases as the pH is shifted away from the pI (charge *vs* pH curve). Therefore, in order to increase the pH at which the heat-induced soluble whey protein/κ-casein complexes should precipitate in milk conditions, one can either aim at increasing their pI or at decreasing their net negative charge throughout the pH range between the pI and the initial pH of milk, pH 6.7. However, it is very important to keep both goals in mind and to always consider the charge *vs* pH curve when modulating the electrostatic properties of the heat-induced whey protein/κ-casein complexes. This should avoid situations where the successful increase of their pI, for instance, is counteracted by the simultaneous increase of their absolute net charge, hence their electrostatic repulsion, too close to the pI. The charge *vs* pH curve also closely depends on the ionic composition of the medium, since, e.g. calcium ions may screen the exposed charges on the whey protein/κ-casein complexes throughout the acidic pH range. As we intend to introduce the modified heat-induced whey protein/κ-casein soluble complexes to a whey protein-free skim milk, the ionic background at the time of measurement will remain constant. However, this effect of the surrounding ions should be kept in mind if the charge and pI of the complexes are to be compared in different mediums.

2.3. Possible methods to modify the surface hydrophobicity of the heat-induced whey protein/κ-casein complexes

As for charge, the protein's genetic variants are a natural source of control over the surface hydrophobicity of the whey protein/κ-casein complexes. Typically, β-lg B is slightly more hydrophobic than A (Allmere et al., 1997) and so is κ-casein B as compared to A (Kyte & Doolittle, 1982). However, as already mentioned, the natural biodiversity of β-lg or κ-casein fails to induce sensible changes neither in their heat-induced complex formation nor in the pH

of acid gelation of the heated milk. Most likely, changes in hydrophobicity across variants do not range widely enough to be efficient.

Because α-la has a significantly lower theoretical hydrophobicity than β-lg or κ-casein (Cayot & Lorient, 1998), it may also be possible to modulate the surface hydrophobicity of the soluble whey protein/κ-casein heat-induced complexes through modulating the protein composition of milk or model systems prior to heating. Graveland-Bikker & Anema (2003) added various amounts of α-la and β-lg to whey protein-free skim milk, then heated the mixture and observed that the gelation pH was increased to a larger extent in the presence of β-lg than α-la. However, the results were discussed in terms of increased number of free thiol groups available for thiol/disulphide polymerisation, rather than in terms of increased surface hydrophobicity of the complexes, when more β-lg was present.

Alternatively, enzymatic or chemical tools have to be considered. Deglycosylation (Cases et al., 2003) or renneting (Gastaldi et al., 1996; Li & Dalgleish, 2006; Niki et al., 2003) have already been performed on the κ-casein in casein micelles or whey protein/κ-casein complexes. The processes are likely to readily expose hydrophobic regions of the κ-casein as they cleave hydrophilic moieties of the protein. However, if hydrophobicity of the casein micelle indeed increases when they are renneted (Peri et al., 1990), this has not yet been demonstrated when renneting the heat-induced whey protein/κ-casein complexes.

Because they catalyze polymerisation reactions that involve charged side groups, transglutaminase or redox enzymes lactase, lactoperoxidase or glucose oxidase also increase the surface hydrophobicity of whey proteins or caseins (Hiller & Lorenzen, 2008). However, oxidoreductases also promote oxidation of thiol groups into intermolecular disulphide bonds (Hiller & Lorenzen, 2008), which could result in changes in formation of the whey protein/κ-casein heat-induced complexes. Gerbanowski et al. (1999) acylated or sulfonylated the lysine's ε-amines of BSA and reported an increase in its surface hydrophobicity as a result of chemical grafting. However, only sulfamidation maintained the secondary structure of the protein. In a different approach, Risso et al. (2000) bound non-anionic hydrophobic Nile Red probes onto casein micelles and reported earlier and faster rennet aggregation. The authors further argued that Nile Red could increase surface hydrophobicity of the casein micelles, without changing

either their net charge or their fractal dimension. As Nile Red can bind at least to β-lg and κ-casein (Sackett & Wolff, 1987), it seems an interesting approach to explore the role of surface hydrophobicity of whey protein/κ-casein soluble complexes in acid gelation, independently of other properties like charge.

Eventually, it may be possible to induce changes in the hydrophobicity of heat-induced whey protein/κ-casein complexes by controlling the temperature of heat treatment. Clearly, unfolding of the whey proteins on denaturation increases their exposed hydrophobicity (Relkin, 1998). This reaction most likely accounts for the high surface hydrophobicity of the heat-induced whey protein complexes (Guyomarc'h et al., 2007b; Jean et al., 2006; Relkin, 1998), albeit Gatti et al. (1995) or Carbonaro et al. (1996) have pointed out that aggregation also engages hydrophobic sites and somewhat limits the gain. Since the work by Dannenberg & Kessler (1988c; 1988d), convergent studies have established that the two-step denaturation/aggregation reaction that yield the whey protein/κ-casein complexes obeys different kinetics below and above ~90°C (Anema & McKenna, 1996; de Jong, 1996; Verheul et al., 1998). Although the common interpretation is that either step is limiting below or above 90°C, de Jong et al.'s calculations (de Jong, 1996) alternatively suggested that unfolding is incomplete at temperatures above 90°C, hence the lower overall activation energy for the reaction. Furthermore, the aggregation step involves more α-la at temperatures above 80°C (Mottar et al., 1989; Oldfield et al., 1998a), which is believed to adversely affect the surface hydrophobicity of the heat-induced complexes at UHT temperatures (Mottar et al., 1989). In his review, de Wit (2009) also explains that at temperatures above 90°C, hydrophobic interactions are prevented due to rearrangement of the water molecules around exposed non-polar regions of the whey proteins. Also, heat-resistant β-sheet and β-strand structures, which confer high local hydrophobicity to the β-lg, eventually unfold at >125°C. The use of high temperature treatments to produce the heat-induced whey protein/κ-casein complexes in milk or model systems may therefore decrease their surface hydrophobicity.

Eventually, Kella et al. (1989) increased the surface hydrophobicity of whey proteins using sulfitolysis of the internal disulphide bonds to loosen their structure, using food-grade reagents.

However, its interest as to increase hydrophobicity on already denatured, disulphide-linked whey protein/κ-casein complexes is yet to be established.

In conclusion, however, one has to bear in mind that manipulating the hydrophobicity of the whey protein/κ-casein complexes without changing their absolute net charge is likely to be a challenge. This challenge is important so that the role of hydrophobic interactions can be discriminated from that of electrostatic ones when using modified heat-induced soluble whey protein/κ-casein complexes to identify the type of interactions that drives the acid gelation of heated milk. In this regard, the method used by Risso et al. (2000) to modify the surface hydrophobicity of casein micelles independently of other properties may prove particularly interesting.

2.4. Possible importance of the κ-casein in the acid gelation functionality of the heat-induced whey protein/κ-casein complexes

Since the addition of heat-denatured whey protein ingredients is effective in increasing the pH of gelation of milk (O'Kennedy & Kelly, 2000; Schorsch et al., 2001; Vasbinder et al., 2004), one could question the importance of the κ-casein in determining the functionality of the heat-induced whey protein/κ-casein complexes. In their study, O'Kennedy & Kelly (2000) pointed out the importance of early interactions between the heat-induced whey protein complexes and casein micelles on gelation pH, the earliest being obtained through co-heating of the two fractions. In milk conditions, this binding occurs naturally through formation of heat-induced whey protein/κ-casein complexes on the surface of the casein micelles (Dalgleish, 1990; Jang & Swaisgood, 1990). Although some heat-induced whey protein/κ-casein complexes are also found in the serum phase of heated milk (Creamer et al., 1978; Guyomarc'h et al., 2003b; Noh et al., 1989; Smits & van Brouwershaven, 1980), they are thought to bind to the surface of casein micelles early on acidification, i.e., prior to destabilisation of the milk (Alexander & Dalgleish, 2005; Donato et al., 2007b; Donato et al., 2007a; Guyomarc'h et al., 2009a). Various authors have further reported that soluble whey protein/κ-casein complexes having a higher proportion of κ-casein were produced on heat-treating milk at slightly increased pH values from ~6.5 up to 8.1 (Anema, 2007; Donato & Dalgleish, 2006; Menard et

al., 2005; Renan et al., 2006). These milks also exhibited an increased pH of acid gelation, typically + 0.2-0.4 pH unit (Anema et al., 2004; Vasbinder & de Kruif, 2003). Among other causes and according to our interpretation, it may be that a higher content in κ-casein renders the soluble complexes more prone to interact with the casein micelles, where κ-casein initially belongs, thus accelerating gelation. This could simply happen because the soluble whey protein/κ-casein complexes are also smaller and more numerous as they contain more κ-casein (Donato & Guyomarc'h, 2009), hence more likely to encounter casein micelles through diffusion. Alternatively, the involvement of κ-casein into the heat-induced whey protein/κ-casein complexes may accelerate acid gelation because formation of the soluble complexes has depleted the casein micelle of its κ-casein and/or because the micelle-bound complexes hinder the stabilising action of the κ-casein. In conclusion, the role of the presence of κ-casein in the heat-induced complexes on the acid gelation of milk still needs to be confirmed. This role could either occur through the possible ability of the whey protein/κ-casein complexes to build facilitated interactions with casein particles in milk during the acidification or thought the removal of κ-casein from the casein micelle. The latter case however relates to the partition of the heat-induced whey protein/κ-casein complexes in milk (reviewed by Donato & Guyomarc'h (2009)), rather than to the controlled modification of soluble whey protein/κ-casein complexes prior to their introduction to milk, as presently reviewed.

In conclusion, the role of the presence of κ-casein in the whey protein/κ-casein complexes formed in heated milk still needs to be evaluated. Following the approach proposed in this review, it could be interesting to modulate the content of the complexes in κ-casein, in order to test whether the presence of this casein influences the acid gelation of milk through facilitated interactions with the casein micelles during the acidification.

3. Properties of the heat-induced whey protein/κ-casein complexes that may affect the strength of acid milk gels

3.1. Structure and mechanical properties of acid dairy gels

In most studies, interactions that build the acid milk gel are investigated using large or low amplitude rheology and confocal or electron microscopy. These tools generally provide an overall view of the strength, lifetime and isotropy of the constitutive interactions, of which it is somewhat difficult to discriminate the respective contributions of the casein micelles and of the heat-induced whey protein/κ-casein complexes. On the basis of our current knowledge of the type of interactions that take part in acid milk gels, one approach could be to modify the heat-induced whey protein/κ-casein complexes as to knock out or enhance their ability to engage a selected type of interaction. If a relationship was found between these variations of the targeted property of the complexes and the mechanical and structural properties of the resulting acid gel, it would indicate the relevance of the selected interaction in affecting the course of the acid gelation of the milk.

In their study of acid milk gels prepared through acidification at 4°C then warm-up, Roefs & van Vliet (1990) and van Vliet et al. (1989) indicated that acid milk gels are essentially structured by electrostatic dipole attraction and by hydrophobic interactions. The fact that skim milk does not gel on acidification at 4°C but gels at higher incubation temperatures shows the implication of hydrophobic interactions in the network (Roefs et al., 1990b). This was further demonstrated by Lefebvre-Cases et al. (1998) or Hinrichs & Keim (2007) who successfully dissociated acid milk gels in SDS. Disulphide covalent bonds have also been evidenced to sustain the acid gels' network (Hinrichs & Keim, 2007) as a result of the formation of heat-induced whey protein/κ-casein complexes on heating the milk. Covalent bonds are thought to account for the increased elastic modulus G' and lower tanδ of acid gels made with heated milk (Guyomarc'h et al., 2003a). Vasbinder et al. (2004) further showed that thiol/disulphide exchanges occur throughout acidification of milk where heat-induced complexes are present and evidenced that disulphide bonds contribute to the increase in G' on gel formation. To a

minor extent, hydrogen bonds have also been suspected to play a role in the gel network (Lefebvre-Cases et al., 1998).

Comparison between unheated milk and sodium caseinate showed that ionic calcium binding does not contribute to the acid milk gel (Lucey et al., 1997b; Lucey et al., 1997c). However, heat-aggregated whey proteins can bind calcium (Halbert et al., 2000; Morr, 1985; O'Connell & Fox, 2001; Simons et al., 2002), but this has yet to be demonstrated in whey protein/κ-casein complexes. It is possible that bound calcium increases the rigidity of the heat-induced whey protein complexes and has consequences on the mechanical properties of the resulting acid gels. But this also has not yet been evaluated. Famelart et al. (2009) suggested that calcium ions as bound to phosphoserine residues in the casein micelles may help strengthen the final acid milk gel. Since the κ-casein molecule has one phosphoserine, this finding may be relevant to the whey protein/κ-casein complexes of heated milk.

Another important feature of dairy gels is their ability to hold water. The presence of heat-aggregated whey proteins clearly decreases syneresis and drainage in acid dairy gels (Guyomarc'h, 2006; Vardhanabhuti et al., 2001). The causes are that, first, denaturation and formation of the heat-induced complexes create new porous structures into which water influxes and immobilises (Denisov et al., 1999; Hinrichs et al., 2004). At the molecular level, unfolding of some secondary structures in the whey proteins induces rearrangement of the hydration water around non-polar groups. Competition of the water molecules for hydrogen interactions within intramolecular β-sheets and α-helices increases, thus increasing bound water and hydration (de Wit, 2009; Denisov et al., 1999). At a larger scale, the heat-induced whey protein/κ-casein complexes finally affect microstructure of the acid gels. Their presence clearly promotes the formation of protein strands between the casein clusters, thus enhancing cohesiveness, connectivity and homogenous porosity, which in turn affect firmness and permeability of the gel (Lucey et al., 1998c; Lucey et al., 1999; Vasbinder et al., 2004). Furthermore, solid, long-time relaxing bonds like those found in the complexes may help prevent intra-particle rearrangement and syneresis, in continuation of the reasoning by van Vliet et al. (1991) or van Vliet & Walstra (1994).

In conclusion, in order to affect the final gel strength of acid skim milk gels, it is hypothesized that the heat-induced whey protein/κ-casein complexes:

- engage the type of interactions that build the gel network (in particular, disulphide or other covalent bonds, hydrophobic attraction, electrostatic attraction)
- sustain the gel's structure by enhancing connectivity between the casein clusters
- immobilise and help immobilise water through controlling porosity of the gel

3.2. Possible methods to modify the protein interactions that build the heat-induced whey protein/κ-casein complexes

By varying the conditions of heat treatment it is possible to modulate the nature of interactions that build the whey protein/κ-casein complexes. For instance, increasing the pH of heat treatment of WPI from 6.0 to 8.0 (i.e. closer to $pK_{SH/S}$- ~9) has been shown to increase the proportion of intermolecular disulphide bonds in the complexes (Hoffmann & van Mil, 1999). In skim milk, an increased proportion of covalent disulphide bonds in the heat-induced whey protein/κ-casein complexes produced at pH up to 7.3 (Donato & Dalgleish, 2006) could partly account for the higher elastic modulus of the resulting acid gels (Anema et al., 2004). On the contrary, blocking the free thiol groups of WPI with N-ethyl-maleimide (NEM) prior to heating yields complexes that fully dissociate in presence of SDS (Hoffmann & Vanmil, 1997). Absence of disulphide bonds within the heat-induced complexes may then account for the low final G' value of skim milk that was heat-treated in presence of NEM, then acidified (Lucey et al., 1998b). Vasbinder et al. (2003b) further showed that thiol/disulphide exchanges continue throughout acidification and significantly contribute to the final gel strength.

Alternatively to variation of the pH of heat treatment, one can modulate thiol/disulphide exchanges in the heat-induced complexes through varying the redox conditions. Reduction of part of the disulphide bonds can be performed using chemical agents like β-mercaptoethanol (Goddard, 1996; Surel & Famelart, 2003) or food-grade alternatives (Kella et al., 1989). By applying electroreduction on whey protein ingredients, Bazinet et al. (1997) could modulate thiol/disulphide-mediated aggregation and reported correlated changes in thermal gelation behaviour. Oxidation to form disulphide bonds can be performed using H_2O_2 (Nabi et al., 2000), by bubbling air or oxygen (Martin et al., 2009; Wanatabe & Klostermeyer, 1976) and can be mediated using enzymes to yield disulphide polymers of the whey proteins (Faergemand et al., 1998).

The addition of extra thiol groups to β-lg either by chemical thiolation (Kim et al., 1990) or genetic engineering (Lee et al., 1993) dramatically increases their ability to polymerise through thiol/disulphide exchange or oxidation. Conversely, it is also possible to generate a thiol-free mutant of bovine β-lg (Jayat et al., 2004) or to use thiol-free porcine β-lg (Gallagher & Mulvihill, 1997) in order to reduce disulphide polymerisation. Lee et al. (1994) reported significant changes in whey syneresis when using genetically modified milk to make yoghurt.

Providing that one of them (generally β-lg or BSA) contains one free thiol group (Calvo et al., 1993; Matsudomi et al., 1993), it is also possible to co-aggregate globular proteins with varying amounts of cysteines and cystines as a mean to affect the acid gelation of milk through changes in the thiol/disulphide ratio of the heat-induced complexes. This could be done either by addition of exogenous globular protein to dairy systems (Famelart et al., 2004; Roesch & Corredig, 2006) or by affecting the natural composition of whey proteins in milk (Graveland-Bikker & Anema, 2003). Kehoe et al. (2007) reported different acid gelation properties of BSA/β-lg heat-induced complexes depending on their ratio and discussed these results in terms of different ability to thiol/disulphide exchanges of the respective proteins. All these approaches indicate that increasing the number of cysteines, rather than cystines, in the reactant proteins enhances acid gel strength.

Interestingly, κ-casein that is specific to the formation of heat-induced whey protein/κ-casein complexes in the milk exhibits enhanced aggregative behaviour when in reduced form (Thorn et al., 2005). Stevenson et al. (1996) demonstrated that the thiolation of caseins other than κ, could help involve e.g. β-casein into heat-induced covalent whey protein complexes. A natural alternative could be to exploit biodiversity in the cysteine content of caseins (Bouguyon et al., 2006).

Despite the high intrinsic energy of covalent disulphide bonds relative to that of low energy interactions (Walstra, 2003a; 2003c), model thiol/disulphide interchange only requires 60-70 kJ mol^{-1} to occur in water (Fernandes & Ramos, 2004). This is in agreement with the estimate of the activation energy of aggregation of the unfolded β-lg in heated milk (Ea ~55 kJ mol^{-1} – de Jong (1996)). Such figures are common for bimolecular/chemical reactions where the breaking and formation of bonds are usually synchronous, so that the overall energy demand is

low (van Boekel & Walstra, 1995; Walstra, 2003c). This suggests that thiol/disulphide exchanges might readily occur, as soon as the reactive sites are in presence. Therefore, another way to act on the thiol/disulphide exchanges in the heat-induced whey protein/κ-casein complexes is to physically control contact between the major reactants, namely, the unfolded whey proteins.

Due to the breaking of a large number of intramolecular hydrogen bonds on unfolding, denaturation of the whey protein results in a large increase in conformational entropy and makes the unfolded protein a thermodynamically unfavourable structure (van Boekel & Walstra, 1995; Walstra, 2003c). Once the protein is unfolded, water molecules organise in the vicinity of the exposed hydrophobic sites as to "exclude" or "cavitate" them from solvent. Intermolecular hydrophobic interactions therefore readily stabilise the denatured proteins. Within such hydrophobic pockets, Fernandes & Ramos (2004) calculated that thiol/disulphide exchanges are dramatically accelerated. Promoting hydrophobic interactions between the protein therefore enhances disulphide polymerisation, in agreement with Oldfield et al. (1998b). Similarly, O'Kennedy & Mounsey (2009) and Mounsey & O'Kennedy (2007) insisted that aggregation of heat-denatured β-lg is largely initiated by non-specific attractive electrostatic and hydrophobic interactions, as favoured at pH close to pI and/or increased ionic strength. When working on humid dairy powders of whey protein isolates, Zhou et al. (2008) observed that the whey protein molecules can undergo partial unfolding and extensive thiol/disulphide exchanges at 35°C providing that the high protein concentration allows close contacts between proteins. In conclusion, in order to control the thiol/disulphide balance in the heat-induced whey protein/κ-casein complexes one could aim at (1) increasing the number of thiols and thiolates (Fernandes & Ramos, 2004) and (2) favouring close contact between proteins.

Intermolecular interactions other than disulphide may also be promoted in the heat-induced complexes. Attractive electrostatic interactions are somewhat significant in protein binding (Xu et al., 1997) and can be promoted if introducing proteins of opposite net charge at the pH of heating. Nigen et al. (2007; 2009) for instance generated electrostatically driven nano-coacervates of apo-α-la and lysozyme at pH 7.5 and 45°C. By comparing β-lg molecules with chemically varied affinities for calcium ions, Simons et al. (2002) estimated unlikely that

calcium ions formed intermolecular bridges in the heat-induced complexes. Therefore, ionic calcium bridges are probably not a lever for controlled changes in the building interactions. Thiol-blocked β-lg has been shown to form non-covalent high molecular mass aggregates on heating model solutions at neutral pH and low ionic strength (Hoffmann & Vanmil, 1997; Mounsey & O'Kennedy, 2007) and Mounsey & O'Kennedy (2007) observed that these aggregates nonetheless yielded as high elastic moduli values on acid gelation as did the control β-lg covalent complexes.

Eventually, the use of polymerisation enzymes like glutaminase may help stiffen the heat-induced whey protein/κ-casein complexes, in the same way it has been shown to increase gel strength in acid skim milk or casein gels (Bonisch et al., 2007; Lorenzen et al., 2002). The studies by Gauche et al. (2009) and Bönisch et al. (2007) suggested that the whey proteins cross-linked with themselves and with the casein micelles using transglutaminase thus paralleled the effect of heat treatment on acid gelation. It further seems that the enzymatic cross-linking of heat-induced whey protein complexes increases the elasticity of the resulting acid gels by increasing rigidity of the complexes (Eissa & Khan, 2005) or by enhancing their interaction with the casein micelles (Bonisch et al., 2005).

On all the above-listed interactions, intra- and inter-particles covalent disulphide bonds are probably the ones that are most susceptible to affect the viscoelastic moduli or the "solid-like" behaviour of the acid milk gels. In our point of view, one choice for future investigation could then be to study the effect of the balance between the content in disulphide bonds inside the heat-induced whey protein/κ-casein complexes and in thiol groups available for the formation of inter-particle disulphide bonds on the acid gelation of milk.

3.3. Possible methods to modify the size of the heat-induced whey protein/κ-casein complexes

Because they are thought to act as spacers or bridges between the casein micelles on acid gelation (Donato et al., 2007a; Lucey et al., 1998b; Lucey et al., 1999), the size of the whey protein/κ-casein heat-induced complexes are quite likely to play a key role in the final microstructure of the acid gel, by affecting, e.g. pore size and connectivity (Kalab et al., 1983;

Parnell-Clunies et al., 1987). Naturally, the serum form of complexes has hydrodynamic diameters ranging 30-100 nm and molecular weight values of ~4.10^6 to 2.10^7 g mol^{-1} (Donato & Guyomarc'h, 2009). In a different field, microparticulated whey protein having sizes of 0.1-2 µm can be used to control firmness and syneresis of yoghurt gels (Janhøj & Ipsen, 2006; Sandoval-Castilla et al., 2004). However, little is known of how the size of the whey protein/κ-casein complexes can affect the properties of acid milk gels.

By increasing the pH of heat treatment of milk from 6.5 to 7.1 (Anema et al., 2004; Rodriguez del Angel & Dalgleish, 2006), acid gels with increased final G' values are produced, although a decrease is found at pH ≥ 7.2. Among other reasons, slightly alkaline pH conditions of heating are known to yield whey protein/κ-casein complexes of smaller size than at pH 6.5 (Creamer et al., 1978; Rodriguez del Angel & Dalgleish, 2006; Vasbinder & de Kruif, 2003). However, the complexes produced at pH 7.1 may also contain more disulphide bonds or active thiol groups, which can both affect the texture of acid gel more than their size (Alting et al., 2003). Similarly, Britten & Giroux (2001) produced smaller complexes in solutions of whey protein isolate at pH 8.5 than 6.5, but failed to correlate size and G' values of the resulting acid gels.

On the other hand, by decreasing the casein to whey protein ratio of milk blends, Guyomarc'h et al. (2003a), Puvanenthiran et al. (2002) or Beaulieu et al. (1999) increased the size of complexes up to 5-fold and obtained acid gels with proportionally increased final G' values. In model whey protein systems, higher protein concentration also promotes formation of larger complexes (Hoffmann et al., 1997; Ju & Kilara, 1998b; Le Bon et al., 1999). In milk, increasing the concentration of only the whey protein using membrane separation techniques promotes their heat denaturation (Mc Mahon et al., 1993; Oldfield et al., 2005), but the likely effect on aggregate size is yet to be established.

In skim milk, increasing the temperature and/or duration of heat treatment increases the extent of denaturation of the whey proteins (Dannenberg & Kessler, 1988c), with proportional consequences on the final firmness and water retention capacity of the acid gel (Anema et al., 2004; Dannenberg & Kessler, 1988a; Dannenberg & Kessler, 1988b). Unfortunately, no similar abacuses have been produced to know how the size of the heat-induced whey protein/κ-casein polymers varies with time and temperature of heating. It is however known from model

whey protein systems that both factors positively affect the size of heat-induced whey protein polymers (Hoffmann et al., 1997; Le Bon et al., 1999; Schokker et al., 2000).

Investigations on the heat-aggregation behaviour of β-lg or whey protein isolate model solutions clearly show that of all factors, the control of electrostatic repulsion between the reactant proteins affects the final size of the heat-induced complexes. As a rule, increasing the ionic strength of the medium decreases the range and intensity of electrostatic repulsion, thus increasing the chance for aggregation, and the size of complexes (Baussay et al., 2004; Caussin & Bouhallab, 2004; Durand et al., 2002; Mahmoudi et al., 2007; Pouzot et al., 2005; Unterhaslberger et al., 2006; Xiong, 1992). Also, having the pH close to the pI of the reactant whey proteins enhances aggregation and yield larger, clustered complexes (Foegeding et al., 2002; Mehalebi et al., 2008; Vasbinder & de Kruif, 2003).

Due to (a) their effective charge screening, (b) the possible induction of conformational changes in the protein and (c) their ability to bind carboxylate groups, calcium ions have long been acknowledged to promote protein aggregation (Ju & Kilara, 1998a; Simons et al., 2002). Parris et al. (1997) or Xiong (1992) reported the enhanced formation of aggregates when increasing ionic calcium concentration in whey or whey isolate prior to heating. Caussin et al. (2003) further showed that heating whey protein isolate in presence of 10 mM $CaCl_2$ yielded larger heat-induced complexes than with 100 mM NaCl, further demonstrating the specific promoting effect of the divalent cation on aggregation. However, this effect is highly dependent on pH (Britten & Giroux, 2001). Simons et al. (2002) and Mounsey & O'Kennedy (2007) found that a stoichiometry of about 0.1 to 0.165 mmol Ca^{2+} per g β-lg is necessary to trigger significant heat aggregation. The conditions for maximum aggregation of whey protein isolates also seem to obey some stoichiometry, albeit the ratio of mmol Ca^{2+} per g protein varies across studies (Ju & Kilara, 1998a; Sherwin & Foegeding, 1997). Simons et al. (2002) explained that heat-induced complex formation could occur only when the repulsive charges born by the carboxyl groups of the whey proteins were shielded by calcium counterions through specific binding.

Studies on the effect of ionic strength or ions have generally been performed on model solutions. To the authors' knowledge, these factors have not been tested in milk as a means to vary the size of the heat-induced whey protein/κ-casein complexes. O'Kennedy & Mounsey

(2009) however reported that the heat-induced aggregation behaviour of the β-lg largely differed whether the protein was in presence of sodium, calcium or phosphate ions; or of mixes of them; or of simulated milk ultrafiltrate. They insisted that the response of the heat-induced formation of whey protein complexes to ionic strength should be dramatically different in a medium where ionic species are in equilibrium with each other, or with the casein micelles. In skim milk whose sodium or calcium contents were varied prior to heat treatment, Farrag et al. (2001) and Ramasubramanian et al. (2008) reported only marginal changes in the resulting acid gelation behaviour. Conversely, dramatic changes are reported when micelle-bound colloidal calcium phosphate is modulated prior to heating (Famelart et al., 2009; Ozcan-Yilsay et al., 2008). All these results suggest that understanding the effect of ionic strength or minerals on the heat-induced whey protein/κ-casein complex formation in milk cannot be anticipated from results obtained in model solutions, and therefore, needs dedicated research.

Eventually, the size of the heat-induced whey protein/κ-casein complexes may be controlled through the use of stabilising molecules. In recent years, research has evidenced that caseins may help stabilise heat-denatured globular proteins from whey or egg in a pattern that resembles chaperone activity (Bhattacharyya & Das, 1999). In the presence of casein material, turbidity of the heated globular protein solution is reduced as a result of decreased aggregate size (Khodarahmi et al., 2008; Koudelka et al., 2009; Matsudomi et al., 2004; Morgan et al., 2005; O'Kennedy & Mounsey, 2006; Yong & Foegeding, 2010; Zhang et al., 2005). In the case of co-heated κ-casein and WPI, smaller and more numerous mixed complexes were produced with increasing proportion of κ-casein (Guyomarc'h et al., 2009b).

Other studies also indicated that ligands or surfactants like arginine, guanidium, lecithins, SDS or sodium laurate could control over-aggregation of heat-induced complexes of milk proteins (Tran Le et al., 2007; Unterhaslberger et al., 2006). Kerstens et al. (2005) reported a peculiar effect of the non-ionic surfactant Tween 20, which promotes formation of µm-large, spherical, size-designed, reversible heat-induced aggregates in concentrated β-lg solutions. Studies that control aggregation of β-lg using coacervation with carbohydrates are out of the scope of the review.

In conclusion, numerous methods are available that could probably be transferred to the soluble heat-induced whey protein/κ-casein complexes to modulate their size. However, only a

very few of them have been applied to study the effect of the size of these specific complexes on the acid gelation of milk. In essence, the available studies rather investigated the effect of the size of larger microparticulated whey proteins, used as fat replacers, in acid milk gels.

3.4. Possible methods to modify the shape of the heat-induced whey protein/κ-casein complexes

Thanks to the large amount of reports that have described the aggregation behaviour of proteins in varying conditions, it now emerges that almost, if not all the proteins are able to alternatively heat-aggregate into branched particulate clusters or into elongated fibres depending on the distribution of functional sites across their exposed surface. As a rule, environmental conditions that promote exposure of local poles of high net charges and/or beta-sheet structure also promotes head-to-tail aggregation, hence fibrillation. Hence, various studies have reported that globular proteins such as ovalbumin (Weijers et al., 2008), β-lg (Bolder et al., 2007; Durand et al., 2002; Ikeda & Morris, 2002; Jung et al., 2008), BSA (Holm et al., 2007) or partially hydrolysed α-la (Otte et al., 2005) can yield fibrils on (even moderate) heat treatment at low ionic strength values and at pH generally ~7.0 or ~2.0 depending on proteins. Elongated particles of β-lg can further be produced in water at pH ~7.0 and ≤50 mM NaCl (Alting et al., 2004; Pouzot et al., 2005). Fibrillation can also occur in acidic solutions of whey protein isolate (Akkermans et al., 2008), yielding somewhat thicker structures than with pure β-lg (Ikeda & Morris, 2002). Bolder et al. (2007) however suggested that although other species were present in solution, β-lg alone is involved in fibrillation when heating WPI. Because of the often acidic pH (~2.0) and orientation constraints, thiol/disulphide exchanges do not seem to contribute to fibril formation, but readily occur when the fibrils are brought to pH 7 to 10 (Bolder et al., 2007). Alternatively, Rasmussen et al. (2007) could produce fibres of β-lg after prolonged exposure to subdenaturing concentrations of chaotropes and showed that their content in disulphide covalent bonds could be modulated depending on whether urea or potassium thiocyanate was used as the chaotrope.

Fibrils in WPI solutions were shown to increase the viscosity of the system and to exhibit shear-thinning behaviour (Akkermans et al., 2008). Veerman et al (2003) compared the ability

of long linear fibrils formed by heating β-lg at pH 2 then brought to pH 7 to yield a gel on addition of $CaCl_2$ to that of random β-lg complexes conventionally produced by heat treatment at pH 7. They showed that the fibrils were able to build a solid gel at much lower protein concentrations than the control complexes. Alting et al. (2004) furthermore mentioned that long fibrillar structures of ovalbumin significantly contribute to the firmness of acid-induced gels. If they could be produced in milk in technological conditions, fibrillar whey protein/κ-casein complexes would most likely help increasing firmness of the acid dairy gels, even if anisotropic structures generally involve less intermolecular thiol/disulphide exchanges (Broersen et al., 2007; Weijers et al., 2008) otherwise desirable to gel firmness (Alting et al., 2004; Alting et al., 2003).

Of all caseins, the κ-casein is also capable of forming fibrils in physiological conditions (pH 7.0-8.0 and 37°C), especially when reduced (Farrell et al., 2003; Leonil et al., 2008; Thorn et al., 2005). In the case of κ-casein, the driving force for fibrillation of the protein is not known, but likely involves intermolecular β-sheet stacking (Thorn et al., 2009). When WPI and κ-casein are heated together at pH 7.0 and 0.1 M NaCl, the heat-induced whey protein/κ-casein complexes turn from particles with a fractal dimension Df of ~2 to linear structures with Df ~1.1 as the proportion of κ-casein exceeds 0.5 g per g WPI (Guyomarc'h and Nicolai, 2009, unpublished results, Figure 7). In milk phase conditions, the protein composition and more specifically the respective proportions of κ-casein and whey proteins may therefore be an efficient means to control the shape of the heat-induced whey protein/κ-casein complexes.

However, as αs or β-caseins inhibit fibrillation of the κ-casein (Thorn et al., 2005) or that of globular proteins, including the whey proteins (see review paper by Yong & Foegeding (2010)), preparation of elongated whey protein/κ-casein heat-induced complexes may first rather be attempted in whey, prior to their addition to milk. Interestingly, Creamer et al. (1978) early reported that the heat-induced whey protein/κ-casein complexes that formed in milk at pH 6.8 were more thread-like than those produced at pH 6.5. Since the involvement of κ-casein into the whey protein/κ-casein complexes of milk seems to increase as the pH of heating increases (Anema, 2007; Donato & Dalgleish, 2006), it is possible that the shape of the complexes may be somewhat controlled in situ through varying the κ-casein to whey protein

ratio of milk or its pH of heating. In the long term, the ratio can also be genetically modified in mammals (Jimenez-Flores & Richardson, 1988).

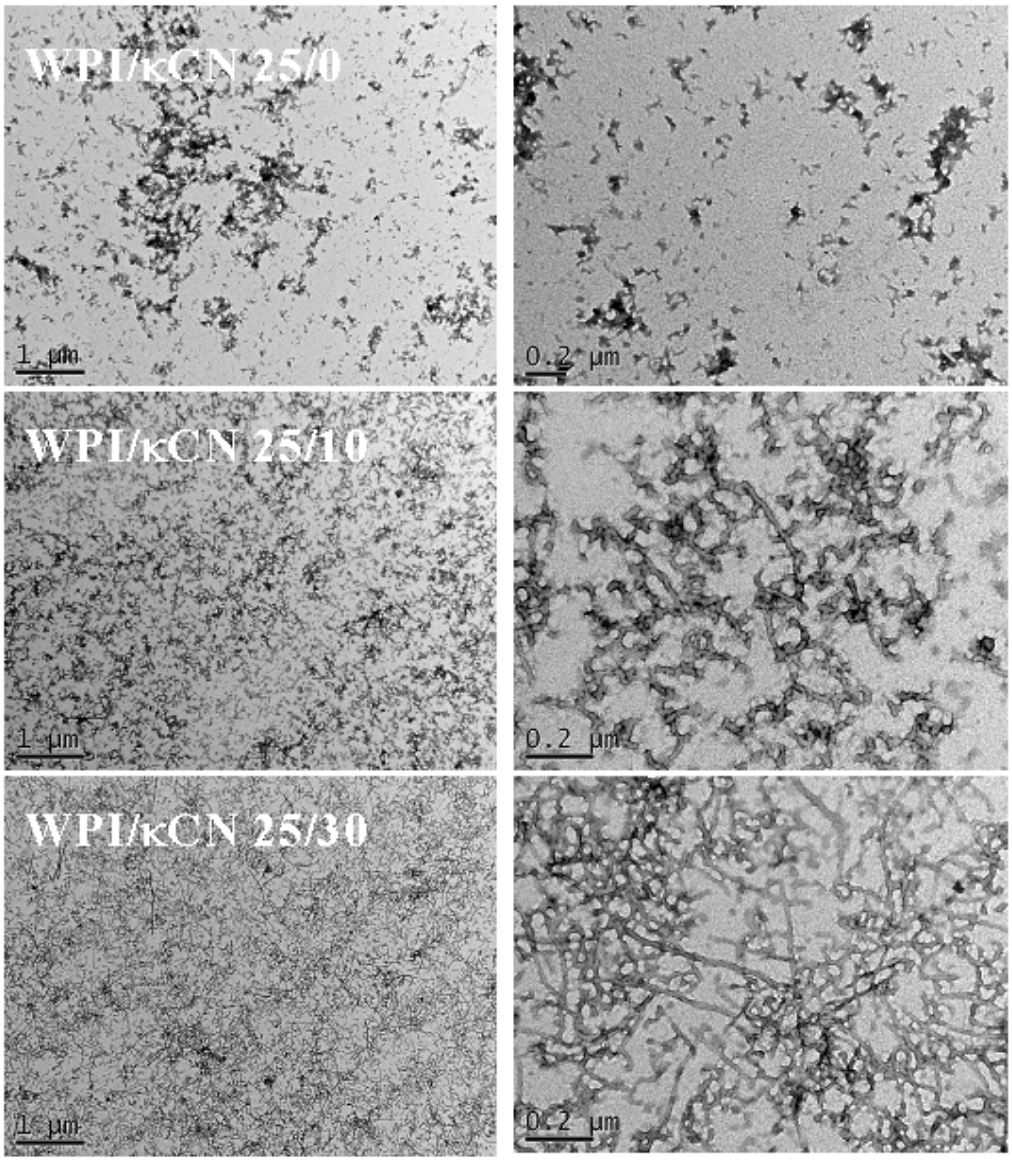

Figure 7 : Transmission electron micrographs of heated (80°C/24h) solutions of 25 $g.kg^{-1}$ whey protein isolate (WPI) and 0-30 $g.kg^{-1}$ isolated freeze-dried κ-casein (κCN) in 0.1 $mol.L^{-1}$ NaCl, pH 7.0, prepared as in Guyomarc'h et al. (2009b)

Although fibrillar complexes may be desirable for texture, there is suspicion that they could be cytotoxic (Hudson et al., 2009). Control of their extension might therefore be useful. Alternative to the involvement of chaperone caseins, the use of chemical grafting of additional reactive sulfhydryl groups onto ovalbumin molecules in solution at pH 7.0 and 0.15 M NaCl

has been shown to yield more branched, amorphous heat-induced complexes as opposed to more fibrillar structures (Broersen et al., 2006). Genetically or chemically modified β-lg with added free thiol groups may be a good means to increase branching of the heat-induced whey protein/κ-casein complexes, but this is yet to be demonstrated. Reduction or disruption of natural disulphide bonds (Bazinet et al., 1997; Kella et al., 1989) may also open routes for branching. Although goat or sheep κ-caseins contain three cysteines, they all seem to be naturally involved into disulphide bonds (Bouguyon et al., 2006) and hence, are unlikely to promote branching.

To the authors' point of view, building acid milk gels in the presence of heat induced whey protein/κ-casein in the form of fibrils that should entangle with each other and provide numerous interaction patches with the casein micelles is very likely to strongly affect the texture of the gels. Although a lot of proteins are able to form fibrils on heat-treatment depending on conditions, to the author's knowledge no attempt has however yet been undertaken to evaluate the effect of heat-induced whey protein or whey protein/κ-casein fibrils on the acid gelation of milk systems.

3.5. Possible methods to modify the water holding capacity of the heat-induced whey protein/κ-casein complexes

It is largely known that the essential factor to improve water holding capacity of acid milk gels is to fully denature the whey protein (Dannenberg & Kessler, 1988a). Conversely to gel firmness, the water holding capacity of yoghurt gel linearly increases with heating, including UHT conditions (Krasaekoopt et al., 2003). At temperatures above 100°C, de Wit (2009) mentions that specific unfolding occurs, possibly as a result of disulphide breakdown that may favour protein hydration (see also section 3.1). It is therefore possible that heat-induced whey protein/κ-casein complexes with less mechanical resistance but higher water holding capacity are formed in UHT conditions.

A higher whey protein to casein ratio in the milk has also been associated with increased water holding capacity of the yoghurt gel (Puvanenthiran et al., 2002; Schorsch et al., 2001) although it is not clear whether the actual composition of the heat-induced complexes, rather

than their size or concentration or shape, is responsible for this change. However, Guyomarc'h et al. (2009b) showed that model heat-induced complexes of whey protein isolate were less dense when involving κ-casein, hence more likely to uptake water through a more porous structure.

The addition of charges (see section 2.2) to the heat-induced complexes may also be a way to improve water binding, providing that the complexes keep destabilising into a gel on acidification. In that sense, the covalent binding of sugars to milk protein has been shown to enhance solubility but also gelation, depending on extent (Oliver et al., 2006). Glycosylation of a peculiar heat-induced whey protein ingredient using dextran molecules was shown to increase its water-binding capacity when re-heated (Lillard et al., 2009). The application of controlled glycosylation of whey protein/κ-casein complexes to yoghurt making is however yet to be investigated.

The presence of counterions that would shield electrostatic repulsion during the formation of thiol/disulphide complexes may be another way to modulate the density of soluble whey proteins/κ-casein complexes. In their review on whey protein functionality, Foegeding et al. (2002) mentioned that gels made of whey protein isolate heated in the presence of salts are denser, coarser and more permeable than at low ionic strength. Pouzot et al. (2005) furthermore showed that heating β-lactoglobulin at neutral pH in the presence of 200 mM NaCl yielded denser β-lactoglobulin covalent complexes than at lower ionic strength values. As another example, Giroux et al. (2010) performed the cold-set precipitation of whey protein isolate preheated at neutral pH and low ionic strength using a pH cycling to pH 5-6 in the presence of increasing concentrations of $CaCl_2$, to produce disulphide complexes of whey proteins having correspondingly increasing density, hence decreased water retention capacity.

On top of increasing polymerisation and gel strength, free thiol groups also seem to be a factor to control syneresis in acid gels. Lee et al. (1994) added recombinant β-lg with two or three free thiol groups to skim milk (<1 g. kg^{-1}) and observed reduced syneresis in the yoghurt produced after heat treatment and fermentation of the modified milk. Possibly, addition of thiol groups promoted branching and hence, reticulated porous complexes that could easily carry water.

4. Perspectives for research

First, this review was wilfully limited to the production of whey protein or whey protein/κ-casein complexes using heat treatment, but other technological means could be considered such as high pressure treatments or dry heating of protein powders. These technologies have been reported to induce whey protein denaturation and complex formation, but with few similarities with the heat treatment (Considine et al., 2007; Ibrahim et al., 1993; Zhou et al., 2008). They could therefore be used in combination with or in substitution to heating, to potentially modulate the properties of the whey protein/κ-casein complexes. The resulting complexes could also be further modified, using a number of the chemical or biological methods listed in the present review.

The present overview of the literature further evidences two major fields for future investigation of the role of the heat-induced complexes in the acid gelation of milk. First, a large number of the reviewed chemical, biological or physical means to modulate the physico-chemical properties of protein material has often been applied on single, native proteins rather than on proteins assemblies like the heat-induced complexes. But modification of the reactant protein is quite likely to affect its aggregation on heat treatment, hence to introduce many other large changes than the initial modification. Therefore, modifying the constituted heat-induced whey protein/κ-casein complexes as a means to vary their interaction properties may prove an interesting scientific strategy.

Second, where as a majority of the studies using modified proteins or modified complexes have investigated the consequences on thermal gels, only a little proportion of them considered acid gelation. Amongst them, only a little number of studies investigated as complex a system as milk (see, e.g. Lieske (1999); Vidal et al. (1998) who modified the casein micelle). In the perspective of better understanding the role of the heat-induced whey protein/κ-casein in the acid gelation of skim milk, we therefore propose to modulate some targeted properties of the complexes, using the presently reviewed methods, then to introduce them into milk where their capacity to establish specific interactions, or to build specific microstructures, will be tested. Should some of these methods be particularly efficient in affecting the acid gelation of milk, their application could then be attempted directly on milk. However, this raises the issue of

correlated changes in the milk, for instance through changes in the composition and equilibrium of milk if ions or pH or exogenous proteins are used to modify the whey protein/κ-casein soluble complexes in situ; or through the simultaneous modifications of the whey protein/κ-casein complexes and the casein micelles if, e.g. chemical grafting is used to modify the soluble complexes in situ. Demonstration of the transferability of the presently reviewed methods, directly to the milk system, is therefore in itself a critical perspective for applicative research.

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II.6.2.3. Bilan de l'article 1

Une synthèse de la revue bibliographique (article 1) est proposée dans le Tableau 2. D'après la littérature, nous avons établi deux catégories de propriétés physico-chimiques des complexes thermo-induits : les propriétés présenties pour modifier le pH de déstabilisation du lait et les propriétés pressenties pour modifier la fermeté des gels acides. Pour chacune des propriétés des complexes, nous avons identifié les moyens chimiques, physiques et biologiques de les moduler par des moyens expérimentaux (scientifiques) ou par des moyens compatibles avec l'alimentation (« foodgrade »).

Tableau 2 : Hypothèse des propriétés des complexes thermo-induits susceptibles de modifier les propriétés de gélification acide de la micelle de caséines, et les moyens pour les moduler, d'après l'article 1 et Famelart et al. (2011)

	Propriété des complexes	**Moyens scientifiques**	**Moyens «foodgrade»**
Modification du pH de déstabilisation de la micelle de caséines	Présence de caséine κ comme point d'ancrage	- composition protéique (protéines purifiées)	- composition protéique (cracking du lait)
	Point isoélectrique	- greffage chimique - ligands ioniques	- addition de protéines globulaires de pI variables - maillardisation - déglycosylation de la caséine κ
	Hydrophobie de surface	- greffage chimique - ligands non-ioniques	- contrôle de la dénaturation - emprésurage
Modification de l'aptitude de la micelle de caséines à former un gel ferme	Pontages disulfures inter et intra	- réduction/oxydation chimique du thiol - greffage ou masquage chimique de thiols - génie génétique	- conditions redox - électroréduction - pH - addition de protéines riches en SH
	Taille	- minéraux et force ionique - ligands	- pH - ratio caséine/protéines de lactosérum - concentration en protéines - lactose et ions
	Forme	- composition protéique (protéines purifiées) - greffage (carboxyméthylation des caséines-κ)	- pH - ratio caséine/ protéines de lactosérum

Objectifs et stratégie du projet

Dans le but de mieux comprendre les mécanismes de formation des gels acides laitiers, l'objectif de cette thèse est d'établir le lien entre les propriétés physico-chimiques des complexes et la nature des interactions complexes-complexes et complexes-micelles établies au cours de l'acidification. L'approche originale de ce travail consiste à utiliser les complexes protéiques thermo-induits comme vecteurs de nouvelles propriétés fonctionnelles des micelles de caséines. En effet, d'après l'étude bibliographique précédente (§II.5), nous pouvons simplifier le modèle de lait chauffé par un mélange de complexes thermo-induits solubles et de micelle de caséines dans la phase soluble du lait. La stratégie de cette thèse peut ainsi être décomposée en plusieurs étapes, illustrées par la Figure 8 :

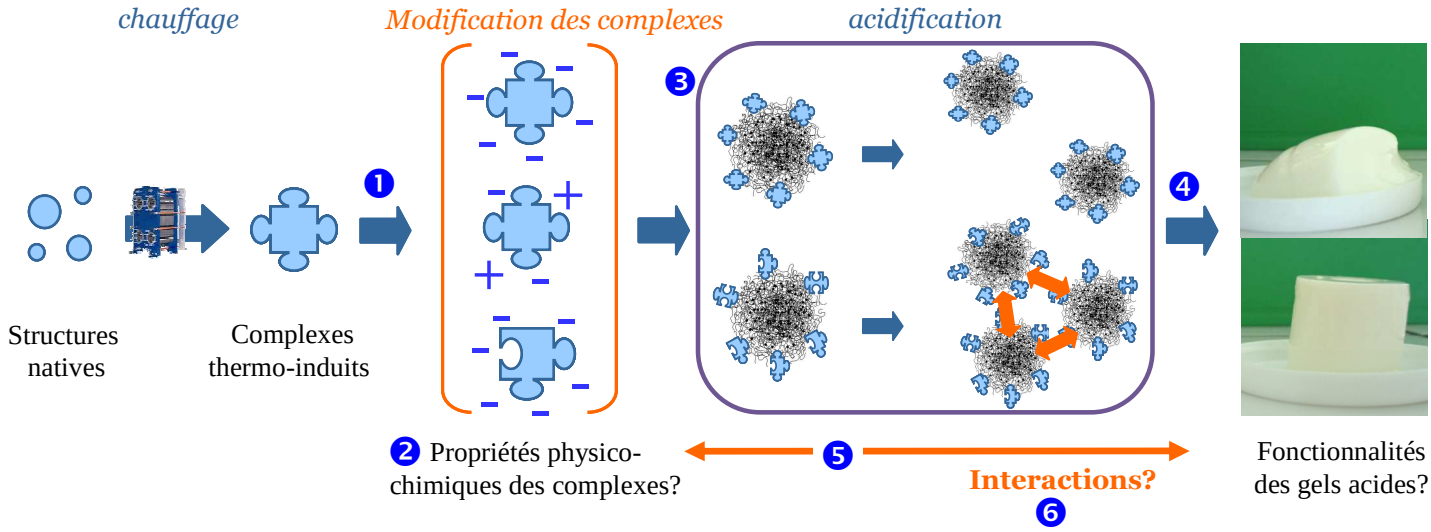

Figure 8 : Schéma de la stratégie adoptée dans l'étude et ses étapes clés

❶ Produire des complexes thermo-induits et modifier une, et une seule, propriété physico-chimique de ces complexes

❷ Caractériser finement les propriétés physico-chimiques de ces complexes

❸ Introduire ces complexes dans un système laitier modèle (i.e. en présence de micelles de caséines et dans un environnement ionique contrôlé) qui sera désigné par la suite lait reconstitué

❹ Acidifier les laits reconstitués : suivre la gélification des laits reconstitués et caractériser les propriétés rhéologiques et structurales des gels acides formés

❺ Etablir les relations entre les propriétés de gélification des laits modèles et les propriétés physico-chimiques modulées des complexes

❻ Conclure sur les interactions établies dans les gels

La stratégie adoptée dans ce projet nécessite la modification d'une seule propriété physico-chimique des complexes à la fois, afin de comparer l'effet de la modification de la propriété étudiée uniquement, par rapport à un témoin. Cette propriété doit également être modulée sur une large gamme afin que son effet sur la gélification du lait soit significatif et permettre de conclure sur les interactions établies dans les gels acides. Pour ces deux raisons fondamentales, nous avons généralement choisi de modifier un complexe déjà constitué, fabriqué selon un traitement thermique standardisé, plutôt que de modifier la composition initiale de l'échantillon (protéines, ions) ou les conditions du traitement thermique (pH, température…). En effet, ces deux alternatives ont généralement des effets considérables sur plusieurs propriétés à la fois. De plus, la modification des complexes a posteriori permet de mieux contrôler l'étendue et les intervalles des niveaux testés, avec de meilleures chances pour conclure statistiquement sur le rôle de l'interaction ciblée par la modification.

Dans ce projet, nous nous sommes focalisés sur les propriétés physico-chimiques des complexes thermo-induits candidates pour influencer le pH de déstabilisation du lait. Le pH de gélification du lait peut tout d'abord avoir des conséquences technologiques. Après le début de la gélification du lait, il est essentiel de ne plus agiter le système, le moindre choc pouvant avoir des conséquences irréversibles sur ce gel fragile. Pour la fabrication de yaourts fermes, l'étape de gélification du lait s'effectue dans les pots. Pour un lait qui gélifie à un pH faible, donc très tardivement, l'industriel bénéficiera de plus de temps pour répartir le lait dans les pots de yaourt, tandis que pour un lait qui gélifie à un pH très élevé, ou très rapidement après son ensemencement, il existe des risques de gélification dans les cuves ou les tuyaux. Nous supposons également que pour un lait qui gélifie à un pH élevé, les gels auront plus de temps pour se réarranger. Enfin, un gel qui gélifie à un pH élevé atteindra peut être un niveau de fermeté suffisant à un pH supérieur à 4.5, le pH du yaourt. Nous pouvons envisager d'arrêter l'acidification avant pH 4.5 ce qui permettrait d'obtenir un yaourt plus doux. Enfin, pour certaines fabrications où le pH du yaourt ne descendrait pas aussi bas que 4.5, à cause d'un fort pouvoir tampon du mélange ou de bactéries lactiques faiblement acidifiantes, ceci permettrait d'obtenir des gels à pH >4.5. Cette situation se rencontre parfois pour des gels acides à fortes teneurs en protéines, par exemple destinés à l'alimentation des seniors.

Les trois propriétés des complexes thermo-induits, identifiées pour avoir un effet probable sur le pH de gélification du lait (Tableau 2), ont été étudiées dans ce projet et correspondent chacune à un chapitre de résultats :

➲ La proportion en caséine-κ intégrée dans les complexes thermo-induits (Chapitre IV)

En effet, la caséine-κ, de part sa localisation à la surface de micelle, est soupçonnée de jouer un rôle de médiation dans l'interaction complexes-micelles au cours du traitement thermique et de l'acidification. Nous supposons ici que la caséine-κ améliore donc les propriétés de gélification acide du lait. Pour moduler la composition en caséine-κ des complexes, une poudre purifiée de caséine-κ a été ajoutée à différentes proportions dans une solution de protéines sériques avant le chauffage. Cette approche a déjà été mise en œuvre pour produire des complexes de protéines laitières (Guyomarc'h et al., 2009b) afin de modifier leur proportion en caséine-κ, mais l'effet de la teneur en caséine-κ dans les complexes sur la gélification acide du lait n'a encore jamais été testé.

➲ Le point isoélectrique (ou pH de charge nulle ou pI) des complexes (Chapitre V)

La gélification des laits crus et chauffés a lieu respectivement à pH 4.8 et 5.5, c'est-à-dire à des pH proches des points isoélectriques de la micelle de caséines et des protéines sériques natives. Les interactions électrostatiques entre particules sont donc un paramètre important de la formation du gel acide. La réduction des répulsions électrostatiques au cours de l'acidification joue probablement un rôle dans la déstabilisation du lait chauffé. Nous souhaitons dans cette partie vérifier la relation entre point isoélectrique des complexes et pH de gélification des laits reconstitués. Parmi les méthodes pour modifier le pI des complexes présentées dans l'article 1, nous avons choisi le greffage chimique d'acide succinique et de méthylamine, pour modifier largement la charge des complexes thermo-induits. Cette modification est opérée après le chauffage afin de réduire son impact sur les autres propriétés des complexes. La succinylation a déjà été réalisée sur des complexes de protéines sériques thermo-induits pour modifier leur pI (Alting et al., 2002) mais son effet sur la gélification acide du lait n'a pas encore été testé. Le moyen de modification du pI des

complexes par la méthylation a été adapté d'une étude sur les protéines végétales (Broersen et al., 2007).

➲ L'hydrophobie de surface des complexes (Chapitre VI)

Les attractions hydrophobes sont suspectées de jouer un rôle dans le rapprochement des particules et donc de participer également à la déstabilisation du lait au cours de son acidification. Nous souhaitons dans cette partie appréhender le rôle des interactions hydrophobes dans la construction des gels acides. Pour les mêmes raisons que la modification du pI, nous avons choisi le greffage chimique de chaines carbonées de différentes longueur par acylation, pour modifier l'hydrophobie des complexes. Ce moyen n'avait pas été listé dans l'article 1 car l'acylation n'a encore jamais été réalisée sur les protéines de lait. Ce moyen a été adapté d'une étude sur les protéines végétales (Gerbanowski et al., 1999).

Chapitre III.

Matériel et techniques expérimentales

III.1. Matériel

III.1.1. Matériaux d'origine laitière

Les compositions des poudres laitières utilisées dans cette étude sont rassemblées dans le tableau ci-dessous :

Tableau 3: Composition des poudres laitières utilisées (en g kg^{-1} de poudre humide)

	Matière sèche	Protéines totales	Caséines	Protéines sériques
Caséine micellaire native (PPCN)	960	910	900	10
Isolat de protéines solubles (WPI)	870	850	60	790
β-lactoglobuline (β-lg)	950	910	0	910
caséine-κ	990	880	880	0

III.1.1.1. Poudre de caséine micellaire native

La poudre de caséine micellaire native (phosphocaséinate natif, PPCN) est produite à l'UMR STLO (Rennes, France). Cette poudre est obtenue à partir de lait écrémé microfiltré à ~50°C à travers une membrane en céramique de taille de pore de 0.1 µm, puis diafiltré, évapo-concentré et enfin séché par pulvérisation (Schuck et al., 1994).

III.1.1.2. Poudre d'isolat de protéines solubles

La poudre d'isolat de protéines solubles (whey protein isolate, WPI) est obtenue à partir du précédant perméat de microfiltration de lait écrémé, contenant les protéines solubles. Ce perméat est concentré par ultrafiltration sur une membrane de taille de pore de 8 kDa, puis diafiltraté et lyophilisé (Fauquant et al., 1988).

III.1.1.3. Ultrafiltrat de lait

Le perméat d'ultrafiltration produit lors de la concentration des protéines sériques est collecté à 5°C puis filtré à 0.1 µm pour garantir sa stérilité. Un agent bactériostatique, l'azoture de sodium (NaN_3) a ensuite été ajouté à 0.2 g L^{-1} pour sa bonne conservation à 4°C.

III.1.1.4. Poudre de β-lactoglobuline native

La poudre de β-lactoglobuline (β-lg) native purifiée a été fournie par la société SOREDAB.

III.1.1.5. Poudre de caséine-κ

La caséine-κ est extraite du lait selon la méthode de fractionnement des caséines mise au point par (Zittle & Custer, 1963). Après la dispersion du mélange de caséines dans de l'urée, les différentes caséines sont sélectivement précipitées par addition de sulfate d'ammonium à différentes valeurs de pH. La fraction de caséine-κ est récoltée, dialysée puis lyophilisée. Sa pureté, déterminée par HPLC, est de 81% des protéines totales, le reste étant constitué de traces des autres caséines.

III.1.1.6. Lait demi-écrémé UHT

Du lait demi-écrémé UHT (marque de distributeur Carrefour) a été utilisé comme solution de dialyse afin de remplacer la phase solvante de certaines suspensions protéiques par celle du lait.

III.1.2. Produits chimiques

Les produits chimiques utilisés au cours de ces travaux ont été achetés chez des fournisseurs (Sigma-Aldrich-Fluka, Riedel de Haen, Interchim) et sont de qualité analytique.

III.2. Techniques expérimentales

III.2.1. Préparation des suspensions de complexes thermo-induits

III.2.1.1. Préparation des suspensions de complexes thermo-induits de WPI et de caséine-κ

Selon la méthode de Guyomarc'h et al. (2009b), des solutions mères de caséine-κ et de WPI ont été préparées à ~100 g kg^{-1} dans une solution de 0.1 M de NaCl et 0.2 g L^{-1} de NaN_3, à pH

7.0. Les suspensions protéiques ont été dialysées contre une solution de 0.1 M de NaCl et 0.2 g L^{-1} NaN_3, à pH 7, dans des boudins de seuil de coupure de 3.5 kDa pour éliminer les sels contenus initialement dans les poudres. La suspension de caséine-κ a ensuite été centrifugée à 59860 g pendant 1 h à 20°C pour éliminer des résidus insolubles et des traces de matières grasses. Le surnageant clair de caséine-κ a été récupéré et filtré sur 0.45 µm (membrane filtrante LMR 25 mm de Labo-moderne). La suspension de WPI, de son côté, a été filtrée sur 0.2 µm (Acrodisc filtre à seringue 25 mm de PALL). La concentration protéique de ces solutions mères a été contrôlée par mesure de l'absorbance à 280 nm en utilisant les coefficients d'extinction massique de 0.93 L g^{-1} cm^{-1} pour la caséine-κ et 1.46 L g^{-1} cm^{-1} pour le WPI.

Des mélanges de 15-20 g kg^{-1} de WPI et 0-10 g kg^{-1} de caséine-κ ont été préparés à partir des solutions mères et d'une solution de 0.1 M de NaCl et 0.2 g L^{-1} de NaN_3 à pH 7.

Les mélanges ont enfin été chauffés dans des tubes fermés en pyrex de 10 mL à 80°C pendant 24 h puis refroidis.

III.2.1.2. Préparation des suspensions de complexes thermo-induits de WPI

Selon la méthode décrite par Vasbinder et al. (2003a), la poudre de WPI a été reconstituée dans de l'eau distillée à 90 g kg^{-1} avec 0.2 g kg^{-1} de NaN_3. La solution a été placée sous agitation pendant 2 h. La solution a été ajustée à pH 7.5 et stockée 24 h à 4°C puis elle a été filtrée sur 0.45 µm.

Les solutions de WPI ont été chauffées dans des tubes fermés en pyrex de 20 mL à 68.5°C pendant 2 h, puis refroidies. Les concentrations protéiques des suspensions de complexes obtenues (ou WPIA) ont été vérifiées par mesure de l'absorbance à 280 nm dans un tampon dissociant et réducteur (8 M d'urée, 0.05 M citrate tri-sodium, 5 g L^{-1} SDS, 0.2 M Tris, pH 7.4 et 10 mM de DTT) en utilisant le coefficient d'extinction massique de 1.46 L g^{-1} cm^{-1}.

III.2.1.3. Modifications des complexes thermo-induits

<u>Principe des réactions chimiques</u>

La modification de l'hydrophobie de surface et/ou du point isoélectrique apparent (pI) des complexes thermo-induits a été réalisée par des réactions de méthylation ou d'acylation sur les

suspensions des complexes précédemment obtenues (§III.2.1.2), diluées au demi dans de l'eau distillée (concentration protéique = 45 g L^{-1}).

Dans le but d'augmenter le point isoélectrique apparent (pI) des complexes, les suspensions de complexes ont été méthylées. La réaction de méthylation des complexes résulte en la neutralisation de charges négatives des résidus carboxyliques par le greffage de la méthylamine (Figure 9A). Les complexes méthylés sont donc moins chargés négativement et leurs charges positives sont surexprimées.

Dans le but d'augmenter l'hydrophobie de surface des complexes, les complexes ont été acylés. La réaction d'acylation des complexes résulte en la neutralisation de charges positives des résidus lysines par le greffage de chaines carbonées (Figure 9B). Les complexes acylés présentent donc une hydrophobie augmentée selon la longueur de la chaine greffée et ils ont perdu une charge positive de la lysine.

Dans le but de réduire le pI des complexes, les suspensions de complexes ont été succinylés. La réaction de succinylation est une acylation particulière, l'acide succinique étant greffé sur les lysines des complexes (Figure 9C). Les complexes succinylés ne présentent pas de modification d'hydrophobie du fait de la charge négative du groupement succinate. Ils ont perdu une charge positive de la lysine et gagné une charge négative par le succinate.

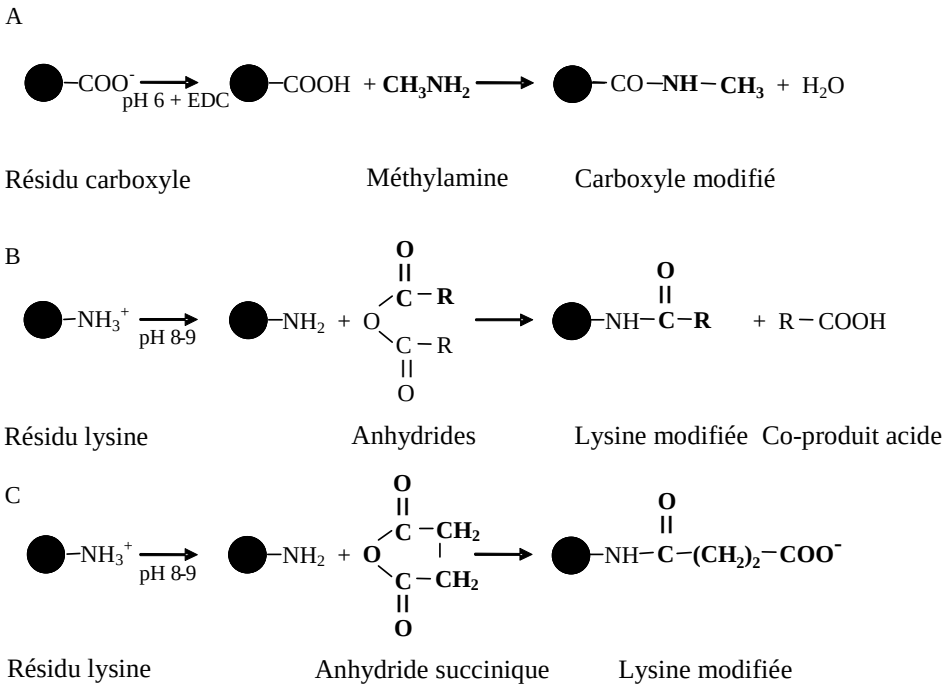

Figure 9 : Diagramme schématique des réactions de méthylation (A), acylation (B) et succinylation (C) des complexes thermo-induits. R = CH_3 avec l'anhydride acétique, R = $(CH_2)_2CH_3$ avec l'anhydride butanoïque et R = $(CH_2)_4CH_3$ avec l'anhydride hexanoïque.

Mise en œuvre

La réaction de méthylation a été réalisée à pH 6 pendant 2 h en présence de 1 M de méthylamine et de 50 à 300 mM de N-3-diméthylaminopropyl-N-éthylcarbodiimide hydrochloride (EDC) selon la méthode décrite par Broersen et al. (2007).

La réaction d'acylation a été réalisée à pH 8-9 pendant 2 h en présence de 20 à 100 mM d'anhydride acétique ($R = CH_3$), butanoïque ($R = (CH_2)_2CH_3$) ou hexanoïque ($R = (CH_2)_4CH_3$) selon la méthode décrite par Gerbanowski et al. (1999).

La réaction de succinylation a été réalisée à pH 8-9 pendant 2 h en présence de 5 à 200 mM d'anhydride succinique selon la méthode décrite par Alting et al. (2002).

Après la réaction, et afin d'éliminer les réactifs en excès et/ou les coproduits, les suspensions des complexes modifiés ont été dialysées dans des boudins de dialyse (6-8 kDa, Medicell International Ltd., London, UK) contre de l'eau distillée ajustée à pH 7.5 et 0.2 g kg^{-1} de NaN_3. Les concentrations protéiques des suspensions ont été vérifiées par mesure de l'absorbance à 280 nm en utilisant le coefficient d'extinction massique de 1.46 L g^{-1} cm^{-1} pour le WPI.

III.2.2. Caractérisation des propriétés physico-chimiques des complexes thermo-induits

Lorsque la méthode le permet, l'analyse des propriétés des complexes thermo-induits a été réalisée dans du MUF ce qui permet de les évaluer telles qu'elles s'exprimeront dans du lait.

III.2.2.1. Mesure de la taille des complexes

a) Mesure du diamètre hydrodynamique

Principe

La diffusion de la lumière par un objet comme des particules protéiques en suspension résulte de la différence d'indice de réfraction entre la particule et son milieu (contraste).

La mesure de la taille des particules en suspension par la méthode de diffusion dynamique de la lumière (dynamique light scattering, DLS) est basée sur la détection de leur mouvement Brownien. La vitesse du mouvement Brownien, déplacement aléatoire des particules dû à l'agitation thermique, dépend de trois facteurs : la température, la viscosité du milieu et la taille

des particules. Le dispositif simplifié est composé d'une source laser monochromatique éclairant une cellule de mesure contenant l'échantillon. Lorsque le laser rencontre les particules de l'échantillon, sa lumière est diffusée dans toutes les directions. Ce rayonnement est recueilli au cours du temps par un détecteur placé à un angle donné de la transmission, puis traité par auto-correlation. L'intensité de lumière au temps t est d'autant moins corrélée avec l'intensité de la lumière à un temps t+dt que la particule a diffusé rapidement pendant dt sous l'effet de l'agitation Brownienne, c'est-à-dire qu'elle est petite. On en déduit le coefficient de diffusion de la particule qui est corrélé à son diamètre hydrodynamique par la relation de Stokes-Einstein :

$$D = \frac{k_B \cdot T}{3\pi \cdot \eta_s \cdot D_h} \qquad \text{(Équation 1)}$$

où D_h est le diamètre hydrodynamique des particules, k_B la constante de Boltzmann ($1.38.10^{-23}$ kg m^2 s^{-2} K^{-1}), T la température absolue (K), η_s la viscosité du solvant (kg m^{-1} s^{-1}) et D le coefficient de diffusion (m^2 s^{-1}).

Mise en œuvre

Le D_h apparent des complexes a été déterminé à l'aide d'un instrument Zetasizer nano ZS (Malvern Instruments, Orsay, France) avec un angle de 173°, sur les suspensions de complexes diluées au 1/20^e dans du MUF à 20°C. Les données ont été converties en distribution de tailles par une modélisation de routine (non-negative least-squares, NNLS). Le mode du pic majeur de la distribution a été pris comme le D_h des complexes de l'échantillon considéré

b) Mesure du rayon de giration et de la masse moléculaire des complexes

Principe

Le rayon de giration (R_g) est la moyenne des distances partant du centre de masse (ou barycentre) d'une structure, pondérés par la masse de matière présente à cette distance. Le calcul du rayon de gyration et de la masse moléculaire des complexes a été réalisé à partir de la mesure de la diffusion statique, ou multi-angles, de la lumière (ou MALLS pour Multi-Angle Laser Light Scattering). Pour une meilleure maîtrise du bruit sur cette mesure très sensible aux

contaminants de grande taille (poussières, micro-bulles…), l'analyse MALLS est généralement faite en aval d'une méthode séparative. Dans ce projet, nous avons utilisé la chromatographie d'exclusion de taille (SEC) qui est couramment utilisée pour séparer les complexes thermo-induits du lait (Guyomarc'h et al., 2010; Jean et al., 2006). Comme nous l'avons dit au paragraphe précédent, la lumière éclairant un échantillon turbide est diffusée dans toutes les directions de l'espace. Si l'intensité de la lumière diffusée varie au cours du temps du fait de l'agitation des particules (diffusion dynamique), elle varie également dans l'espace du fait des positions relatives des éléments diffusants (particules, sous-structures des particules…) entre eux à l'instant t. Pour simplifier l'analyse, l'espace est réduit aux deux dimensions d'un plan confondu avec l'axe de la lumière incidente. Dans ce plan, la distance entre deux éléments diffusants de l'échantillon varie selon l'angle d'observation θ. Ainsi, la somme des ondes diffusées par ces deux éléments va varier en fonction de l'angle, du fait de ce déphasage (Figure 10).

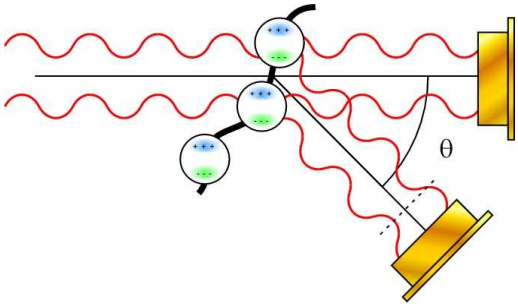

Figure 10 : Illustration du déphasage entre les ondes diffusées par 2 éléments diffusants d'un échantillon, sur l'axe d'observation à angle θ, d'après Wyatt Technology (manuel technique)

Si des motifs répétés existent dans l'échantillon, tels que la présence de particules de tailles similaires et réparties uniformément dans l'espace, ou la présence de sous-unités dans ces particules, la dépendance de l'intensité de la lumière diffusée à l'angle d'observation présentera une forme caractéristique dont l'analyse permettra de déduire des longueurs caractéristiques telles que le rayon de gyration moyen des particules en suspension, ou la taille des sous-unités qui les constituent.

Comme l'intensité de la lumière statique diffusée à un instant t et à un angle donné résulte de la somme des ondes diffusées par N éléments selon leurs positions relatives, elle dépendra également de la concentration, de la masse et de l'indice de réfraction de ces éléments dans le

volume de l'échantillon éclairé par le laser. Ainsi I (θ), généralement transformée en « rapport de Rayleigh » R(θ), s'exprime par :

$$I(\theta) \approx R(\theta) \propto M \cdot c \cdot K \cdot P(\theta) \qquad \text{(Équation 2)}$$

où M est la masse moléculaire apparente en poids (M_w, en $g.mol^{-1}$), *c* la concentration (en $g.ml^{-1}$ ou $g.cm^{-3}$), P(θ) le facteur de forme (sans unité) et K* le facteur de contraste dans lequel intervient l'incrément d'indice de réfraction du soluté dn/dc dans un milieu d'indice n_0 (en $cm^3.g^{-1}$) et le nombre d'Avogadro Na :

$$K^* = \frac{4\pi^2 \cdot n_0^2}{N_A \cdot \lambda_0^4}\left(\frac{dn}{dc}\right)^2 \qquad \text{(Équation 3)}$$

K* s'exprime en $mol.cm^3.g^{-2}$.

L'incrément dn/dc s'obtient expérimentalement par la mesure de l'indice de réfraction n d'une solution en fonction de concentrations croissantes de soluté. En solution suffisamment diluée, n est en effet linéairement lié à la concentration et dn/dc est une constante. Sa valeur est de 0.183 $cm^3.g^{-1}$ pour les protéines à la longueur d'onde de 658 nm et à 25°C. Le dn/dc est aussi utilisé pour mesurer la concentration *c* en tous points du chromatogramme SEC grâce à un réfractomètre différentiel (la cellule de référence contient la phase mobile).

Le facteur de forme P(θ) introduit la dépendance angulaire dans l'expression de l'intensité de la lumière diffusée I (θ) et est fonction du rayon de gyration. Dans des situations de faibles concentrations où les interactions sont négligeables, ce qui est le cas dans l'éluât de SEC, quand θ → 0, P(θ)→ 1 et **R(θ)/K*c → M**

La masse moléculaire est ainsi obtenue par l'extrapolation vers l'ordonnée à l'origine du plot de R(θ)/K*c en fonction de θ (Figure 11). Le rayon de giration (R_g) est quant à lui obtenu à partir de la pente de la représentation de K*c/R(θ) en fonction de $\sin^2(\theta/2)$ (Figure 11).

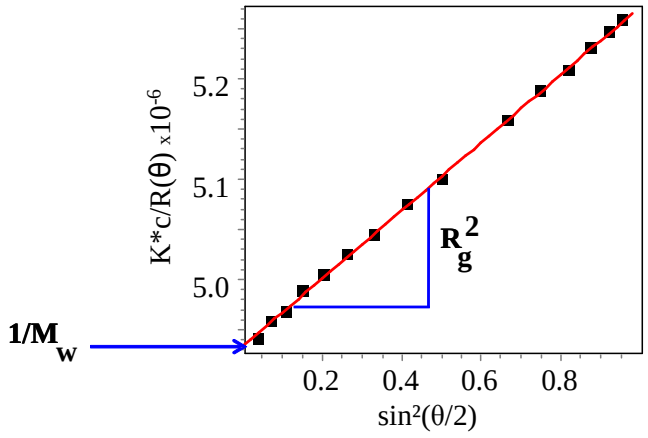

Figure 11 : Mesure de la masse moléculaire et du rayon de gyration

Mise en œuvre

Le montage instrumental (Figure 12) comporte :

- une chaine chromatographique équipée d'une colonne d'exclusion stérique (size exclusion chromatography, SEC),

- un détecteur UV,

- un équipement laser de diffusion statique de lumière à angles multiples (multi-angle laser light scattering, MALLS) comprenant 18 détecteurs de lumière (LS),

- un réfractomètre différentiel (RI).

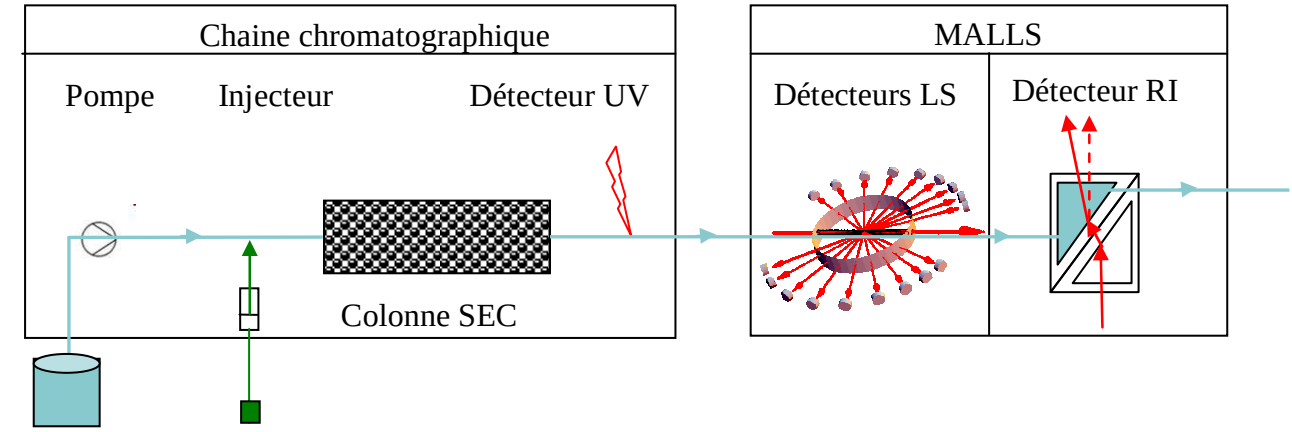

Figure 12 : Schéma du montage expérimental de la chromatographie d'exclusion de taille couplée à un laser de diffusion statique de lumière multiple

Une colonne Sephacryl S-500 Hi-Prep 16/90 (Amersham Biosciences, Orsay, France) a été utilisée pour la séparation des particules de l'échantillon selon leur taille (SEC). La phase mobile était une solution 0.1M Tris, 0.5M NaCl et 10 mM NaN3 à pH 7, filtrée sur 0.1 µm et dégazée. Cent µL d'échantillon ont été injectés, le débit d'élution a été fixé à 0.5 mL min-1 et l'absorbance a été mesurée à 280 nm. Le détecteur de diffusion de lumière LS DAWN-Heleos II était équipé d'une cellule K5 et d'un laser He-Ne à 658 nm (Wyatt Technology, Santa Barbara, CA, USA). Le détecteur d'indice de réfraction RI Optilab rEX était équipé d'un laser à 685 nm (Wyatt Technology). Les données des détecteurs ont été collectées et analysées avec le logiciel Astra v.5.3.4.11 (Wyatt Technology). Les valeurs de Mw et Rg pour le complexe considéré ont été prises au temps d'élution correspondant à 50% de l'aire totale du pic des complexes (Figure 13).

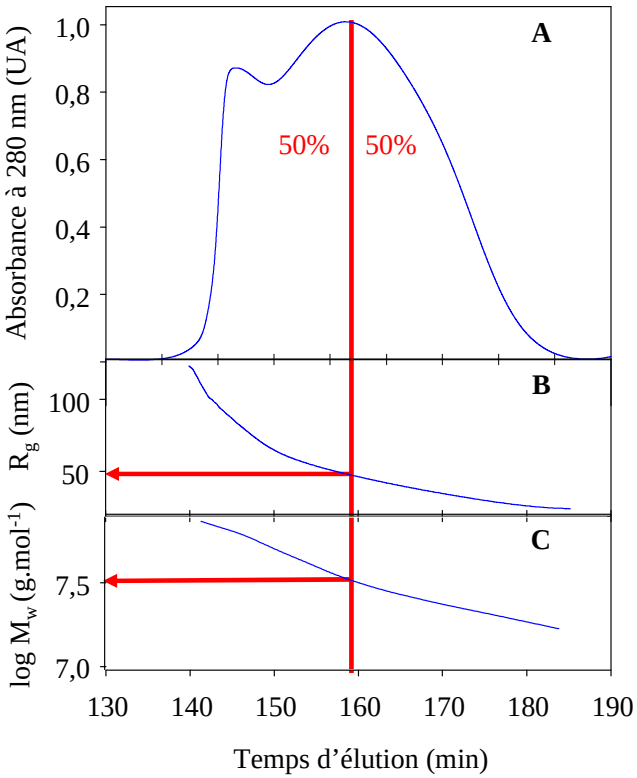

Figure 13 : (A) Chromatogramme d'une solution de complexes injectée dans une colonne chromatographique d'exclusion de taille (SEC). Le temps d'élution pour la détermination du rayon de giration (R_g) (B) et de la masse moléculaire (M_w) (C) est choisi à 50% de l'aire du pic d'absorbance correspondant aux complexes

III.2.2.2. Mesure du point isoélectrique apparent des complexes

Le point isoélectrique apparent (pI) d'une molécule est le pH où cette molécule présente une charge électrique résiduelle nulle. Nous avons déterminé le pI des complexes par zétamétrie, c'est-à-dire en mesurant la mobilité électrophorétique (mb) des complexes à différentes valeurs de pH. La mb est en effet proportionnelle à la charge nette, et lorsque la charge nette des complexes est nulle, la mb vaut 0 µm cm V^{-1} s^{-1} (Figure 14). Les mesures ont été effectuées sur le même instrument Zetasizer que pour la mesure de taille en DLS à 50 V, 20°C et 633 nm. Les suspensions de complexes ont été diluées au 1/10^{e} dans de le MUF acidifié à différents pH. Du chlorure de calcium a été ajouté au MUF acidifié aux concentrations théoriques d'un perméat de lait écrémé acidifié aux mêmes valeurs de pH, en utilisant le logiciel MILK SALT GLM développé par Mekmene et al. (2009).

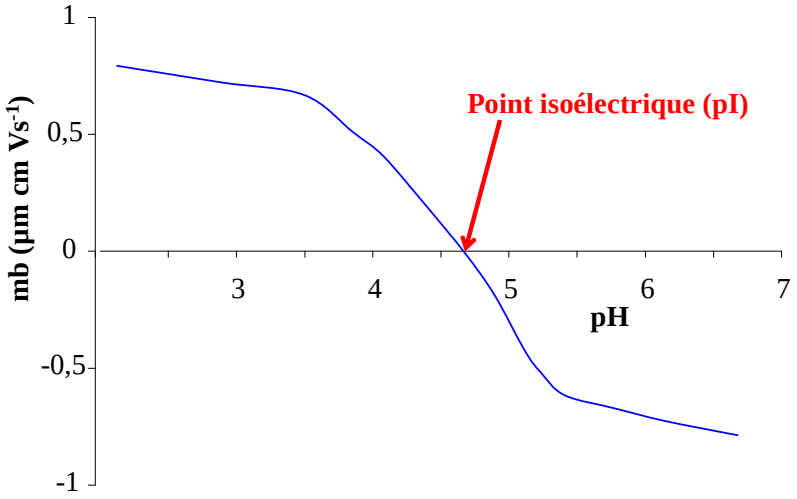

Figure 14 : Détermination du point isoélectrique apparent (pI) des complexes par la mesure de la mobilité électrophorétique à différents pH

III.2.2.3. Mesure de l'hydrophobie de surface des complexes

L'hydrophobie de surface des complexes thermo-induits a été déterminée par la méthode de fixation d'une sonde fluorescente. Deux sondes hydrophobes ont été utilisées : l'acide 8-anilino-1-naphtalène sulfonique (ANS) et le Prodan. Les mesures ont été réalisées à l'aide d'un spectrophotomètre LS50B (Perkin Elmer, Saint Quentin-en-Yvelines, France). Lorsqu'une sonde interagit avec un site hydrophobe, en réponse à une excitation à une longueur d'onde d'excitation ($\lambda_{excitation}$), il y a émission de fluorescence à la longueur d'onde d'émission ($\lambda_{émission}$) (Figure 15). L'intensité de fluorescence émise maximale (If_{max}) peut être relevée. Elle est proportionnelle au nombre de molécules de sondes fixées.

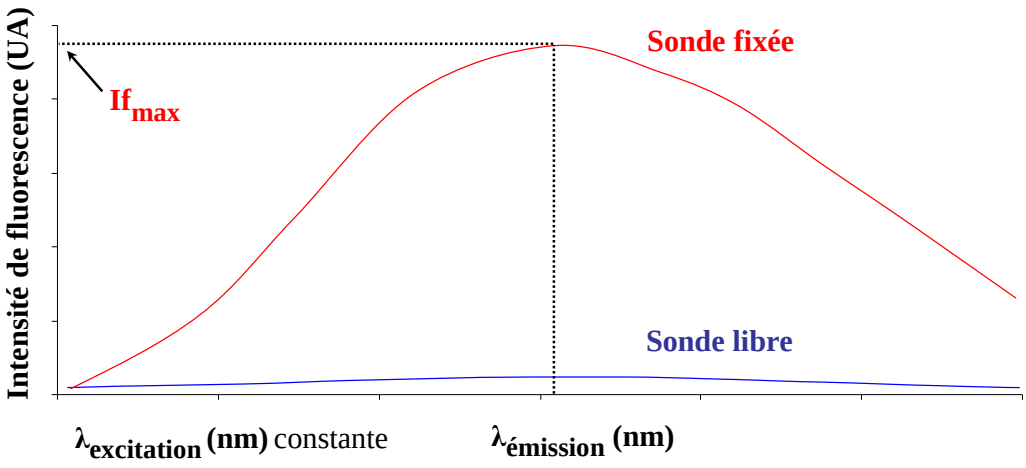

Figure 15 : Spectre d'émission d'une sonde fluorescente libre ou fixée.

a) ANS

Selon la méthode de fixation de l'ANS, le dosage de l'hydrophobie de surface des complexes a été réalisé à une concentration en protéine fixe (1.5 µg L^{-1}) dans une solution tampon maléate (1 M) - NaOH (0.25 M) à pH 6.7, à laquelle 0-40 µM d'ANS a été ajouté. L'absorbance à 390 nm ne doit pas excéder 0.1-0.15, valeur limite supérieure de linéarité pour l'intensité de fluorescence émise par le complexe ANS-protéine. La longueur d'onde d'excitation a été fixée à 390 nm et l'émission a été mesurée à 480 nm avec une largeur de fente d'émission et d'excitation de 2.5 nm.

L'augmentation de fluorescence (F) avec l'augmentation de la concentration d'ANS ([ANS], en µM) qui traduit la fixation de la sonde, c'est-à-dire la titration des sites hydrophobes, a été modélisée par l'équation de Michaelis-Menten (Bonomi et al., 1988):

$$F = \frac{F_{max} \times [ANS]}{K_d + [ANS]} \qquad \text{(Équation 4)}$$

où F_{max} est le maximum de fluorescence à la saturation en ANS (Figure 16) et est proportionnel au nombre de sites hydrophobes accessibles à la sonde sur la particule. K_d est la constante de dissociation du complexes ANS-protéine ou concentration en ANS à $F_{max}/2$ (Figure 16).

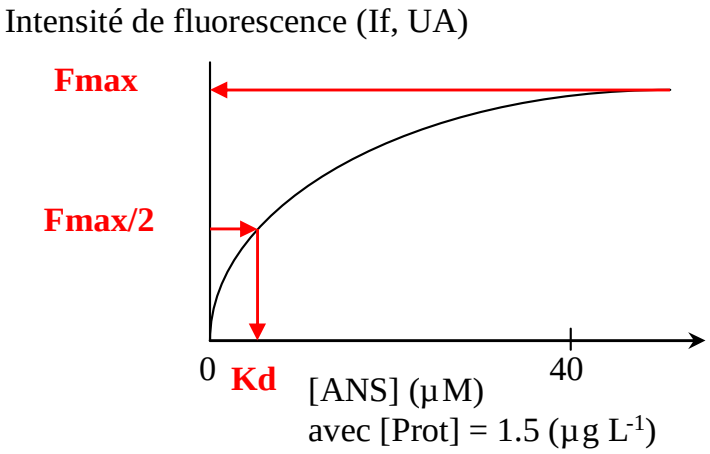

Figure 16 : Paramètres issus de la modélisation de Michaelis-Menten pour déterminer l'hydrophobie de surface des complexes par la méthode de fixation de l'ANS

L'hydrophobie de surface des complexes peut être évaluée par le calcul de l'index d'hydrophobie de surface des protéines (protein surface hydrophobicity index, PSH), précédemment décrit par (Erdem, 2000):

$$PSH_{ANS} = \frac{F_{max}}{K_d \cdot [prot]} \qquad \text{(Équation 5)}$$

où [prot] est la concentration en protéines de la solution en µmol L^{-1}.

b) Prodan

Selon la méthode de fixation du Prodan (Alizadeh-Pasdar & Li-Chan, 2000), l'hydrophobie de surface des complexes a été déterminée avec une concentration constante en Prodan (1.75 µM), et en ajoutant le Prodan aux complexes à différentes concentrations ([Prot]=0-0.1 g kg^{-1}). Les complexes ont été dilués dans un tampon phosphate (0.1 M d'acide citrique, 0.2 M de Na_2HPO_4, 0.02 % de NaN_3 à pH 7). L'intensité de fluorescence, If, a été mesurée pour des longueurs d'onde d'excitation et d'émission respectivement de 365 nm et 465 nm avec une fente de 5 nm, après 15 min de contact, à l'abri de la lumière. La valeur de l'hydrophobie, PSH_{Prodan} a été mesurée par la pente de la droite If = f([Prot]) (Figure 17).

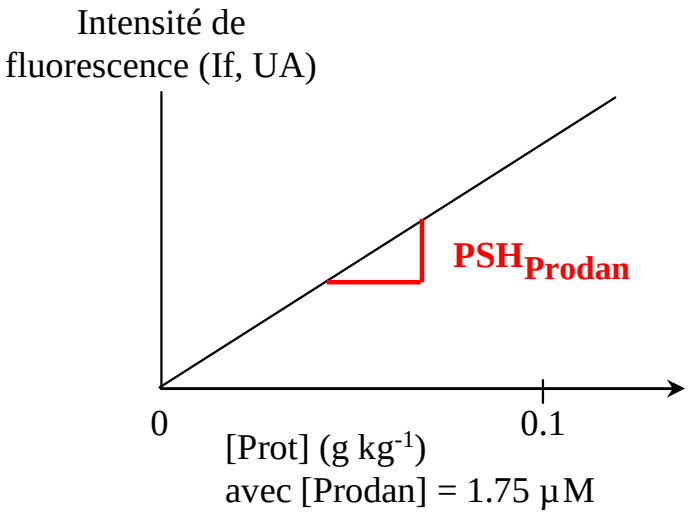

Figure 17 : Détermination de l'hydrophobie de surface des complexes thermo-induits (PSH_{Prodan}) par la méthode de fixation du Prodan

III.2.2.4. Dosage des résidus cystéines

Nous distinguons 3 différents types de résidus cystéines qui peuvent être dosés par l'acide 5,5'-dithio-bis-2-nitrobenzoïque (DTNB) (Ellman, 1959) : les résidus cystéines libres accessibles ou de surface (SH de surface), les groupements résidus cystéines libres totaux (SH totaux) et les résidus cystéines totaux, comprenant les SH totaux et les cystéines impliquées dans des ponts disulfures (SH+SS). La réaction entre le DTNB et les résidus cystéines produit du 2-nitro-5-thiobenzoate (TNB^{2-}) soluble et coloré que l'on peut doser à 412 nm avec un spectrophotomètre (Uvikon 922, Kontron France, Paris, France). Les résultats ont été exprimés en µmol de SH par g de protéine en utilisant les coefficients d'extinction molaire de 18000 et 19000 L mol^{-1} cm^{-1}, obtenus par une gamme de solutions de cystéine pure de concentrations

connues entre 0 et 0. 0.028 mM, repectivement diluées dans un tampon non dissociant et dissociant.

Les concentrations en SH de surface ont été déterminées sur les suspensions des complexes diluées à ~ 5 g kg^{-1} dans un tampon non dissociant (0.2 M Tris-HCl, pH 7.4). Un mL of DTNB à 1 g.L^{-1} a été ajouté à 9 mL d'échantillon dilué. Après incubation à température ambiante pendant 5 min, du sulfate d'ammonium à 0.6 g mL^{-1} a été ajouté pour précipiter les protéines et arrêter la réaction. Après filtration sur du papier Whatman 40, le TNB^{2-} a été récupéré dans le filtrat clair puis dosé à 412 nm. La cellule de référence contenait 9 mL de tampon non dissociant et 1 mL de DTNB.

Les concentrations en SH totaux ont été déterminées sur les suspensions des complexes diluées à ~ 2 g kg^{-1} dans un tampon dissociant (8 M d'urée, 0.05 M citrate tri-sodium, 5 g L^{-1} SDS, 0.2 M Tris, pH 7.4). Trente cinq µL de DTNB (à 1 g L^{-1}) ont été ajoutés à 1 mL d'échantillon dilué. Le dosage du TNB^{2-} a été immédiatement réalisé à 412 nm. La cellule de référence contenait 1 mL de tampon non dissociant et 35 µL de DTNB.

Les concentrations en SH+SS ont été déterminées sur 100 µL des suspensions de complexes diluées à ~ 30 g kg^{-1}. Les complexes ont été dissociés et réduits par 20 µL mL^{-1} de β-mercaptoethanol et 1 mL d'urée 10 M, et incubés à 37°C pendant 1 h. Pour éliminer l'excès de β-mercaptoethanol, les protéines ont été précipitées par l'ajout de 5 mL d'acide trichloroacétique (TCA) à 12% et récupérées par centrifugation (5000 g, 10 min, 20 °C) et ceci 2 fois successivement. Les protéines ont ensuite été re-solubilisées dans 3 mL de tampon dissociant et 105 µL de DTNB à 1 g L^{-1} ont été ajoutés. Le dosage du TNB^{2-} a été immédiatement réalisé à 412 nm. La cellule de référence contenait 1 mL de tampon non dissociant et 35 µL de DTNB.

La teneur en résidus cystéine a également été déterminée par la méthode chromatographique décrite par Croguennec et al. (2001). Les solutions protéiques ont été oxydées par l'acide performique puis hydrolysées avec 6 M d'HCl à 110 °C pendant 24 h selon Moore (1963). Les cystéines ont ensuite été dosées par un analyseur Pharmacia-LKB (Alpha Plus).

III.2.2.5. Détermination du taux de modification des lysines

Le taux de modification des complexes a été déterminé par une méthode dite OPA (ortho-phthaldialdehyde) (Church et al., 1983). Cette méthode permet de déterminer la quantité de groupements aminés NH_2 libres dans une protéine. Le dosage est basé sur la réaction entre l'OPA et le groupement amine, qui en présence de 2-diméthylaminoéthanothiol hydrochloride (DMA) permet la production d'un dérivé iso-indole, composé aromatique qui absorbe la lumière à 340 nm. Ainsi le taux de modification a été calculé en déterminant la concentration en NH_2 libre des complexes témoins (NH_2^0) et des complexes modifiés (NH_2^m) selon la relation suivante (Gerbanowski et al., 1999) :

$$\text{Taux de modification (\%)} = \frac{[NH_2^m] - [NH_2^0]}{[NH_2^0]} \times 100 \qquad \text{(Équation 6)}$$

Le coefficient d'extinction massique 0.034 L g^{-1} cm^{-1} a été déterminé par calibration sur de la leucine.

III.2.2.6. Détermination de la masse des protéines natives modifiées

Le taux de modification des protéines modifiées par méthylation, qui n'a pas été déterminée par la méthode OPA précédente, peut être évalué par une méthode alternative de chromatographie liquide haute performance en phase inverse (RP-HPLC) couplée à la spectrométrie de masse. Cette méthode ne nous permet pas d'analyser directement les complexes thermo-induits modifiés, du fait de leur trop grande taille (70-130 nm). Pour y pallier, la dissociation/réduction des complexes a été tentée mais a posé des problèmes d'adaptation de la méthode. Nous avons donc choisi d'analyser les protéines natives (WPI et β-lg) modifiées et non modifiées par méthylation et succinylation afin d'observer l'augmentation de la masse des protéines natives suite à ces greffages. Dans l'hypothèse où les réactions chimiques se dérouleraient de façon similaire sur les protéines et les complexes thermo-induits, nous pourrons conclure sur le taux de modification des complexes.

Principe

Les protéines sont isolées en chromatographie liquide en phase inverse (HPLC, Reverse Phase-High Performance Liquid Chromatography), puis le pic d'élution de la β-lg est introduit dans le spectromètre de masse et la masse moléculaire de chacune des populations de protéines présente peut être déterminée. Les protéines sont ionisées par nébulisation, puis accélérées sous l'action d'un champ électrique et séparées selon leur rapport masse/charge (m/z) sous l'action couplée d'un champ électrique et d'un champ magnétique. Chaque ion de m/z connu est détecté et le courant ionique correspondant est amplifié, puis mesuré au niveau d'un multiplicateur d'électrons, ce qui permet pour chaque m/z d'obtenir un signal analysable et quantifiable.

Mise en œuvre

Les solutions de WPI et de β-lg modifiés et non modifiés ont été analysées en spectroscopie de masse LC/MS. Les échantillons ont tout d'abord été dilués à 9 g L^{-1} dans une solution d'acide trifluoroacétique 0.01% (v/v) et d'acide méthanoique 0.8% (v/v).

Une séparation préliminaire de la β-lg a été réalisée en HPLC avec une chaine HPLC Agilent HP 1100 (Agilent Technologies, Massy, France) et une colonne Luna C18(2) (Le Pecq, France) de 2 mm de diamètre interne, 10 cm longueur contenant des grains de diamètre 3 µm. Les solutions tampons A et B sont respectivement 0.106% (v/v) d'acide trifluoroacétique et 80% (v/v) d'acétonitrile avec 0.1% (v/v) d'acide trifluoroacétique. La séparation a été réalisée à 40°C avec une augmentation du gradient de tampon B entre 35 et 80% (v/v) en 15 min. Le débit était de 0.25 mL min^{-1}. Douze µL d'échantillon (~100 µg de protéines) ont été injectés et la détection de l'absorbance a été suivie à 214 nm.

Le pic de la β-lg a ensuite été injecté dans un spectromètre de masse à triple quadripôle API-III^{+} Perkin-Elmer (Sciex Instruments Thornhill, Canada). L'ionisation avait lieu à pression atmosphérique dans une source électrospray, sous l'action d'un champ électrique, en mode positif, entre le capillaire contenant l'éluat (4800V) et une contre-électrode (650V) et l'action d'un gaz nébulisateur (l'air à ~0.5 MPa) et un contre-courant d'azote de 1.2 $L.min^{-1}$ entre la source d'ionisation et l'orifice d'entrée dont le potentiel électrique était fixé à 80 V. Le triple quadripôle sélectionnait successivement les différents ions de m/z allant de 300 à 2350 Da, par

intervalles de 0,5 Da, et effectuait un balayage de m/z toutes les 0,5 s. Les données ont été analysées au moyen du logiciel BioMultiView Sciex. L'appareil a été calibré en m/z au moyen de complexes d'ammonium et de polyéthylène glycol de m/z connus.

III.2.2.7. Composition protéique des suspensions protéiques, des suspensions de complexes et des complexes isolés

Les éluats de la SEC (§III.2.2.1.b) contenant les complexes thermo-induits ont été dialysés extrêmement contre de l'eau (6-8 kDa) afin d'éliminer tous les sels de la phase mobile, puis lyophilisés. Les suspensions de protéines natives, les suspensions de complexes et les complexes isolés ont été dilués à ~30 g L^{-1} dans un tampon de dissociation (7M d'urée, 20 mM de bistripropane à pH 7.5 additionné extemporanément de 7µL mL^{-1} de β-mercaptoéthanol) et incubés 1 h à 37°C. Trente µL d'échantillons ont ensuite été injectés sur une colonne d'HPLC selon la méthode décrite précédemment (§III.2.2.6). Les pics du profil d'élution ont été identifiés d'après la calibration de Jean et al. (2006) (Figure 18).

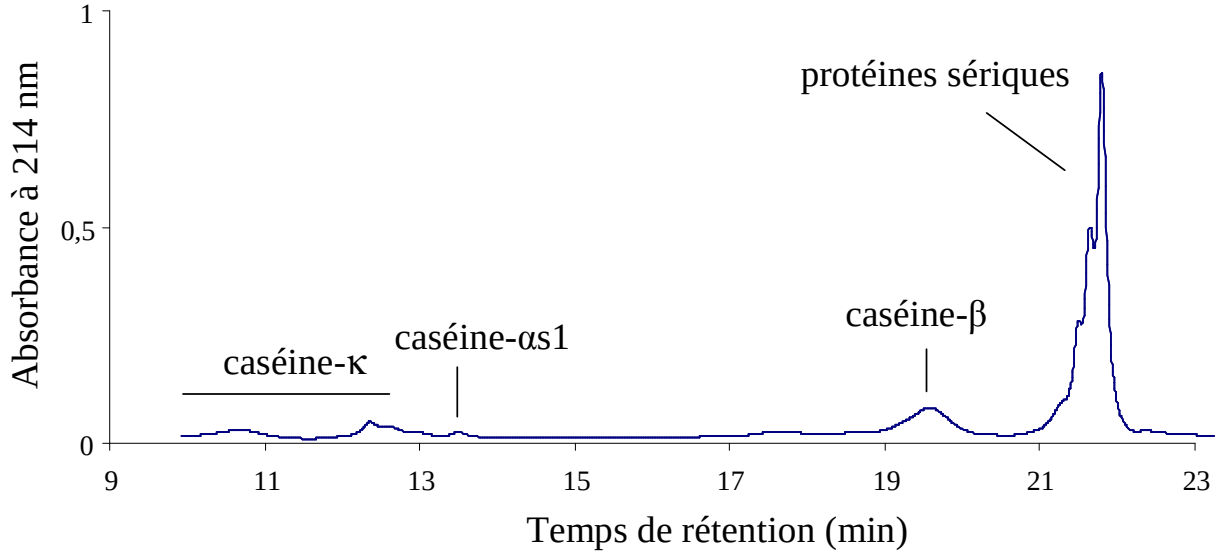

Figure 18 : Profil d'élution obtenu, par chromatographie liquide en phase inverse (HPLC), d'une solution de protéines sériques

Les aires sous les pics identifiés ont été intégrées afin d'établir la proportion en caséine et en protéines sériques des échantillons et, par comparaison entre le mélange chauffé et les complexes purifiés, la proportion des protéines intégrées dans les complexes lors du chauffage.

III.2.2.8. Caractérisation de la structure secondaire des complexes thermo-induits

Principe

La spectroscopie infrarouge à transformée de Fourrier (IRTF) est une méthode d'analyse rapide, non destructive, applicable à de nombreux produits. Le domaine spectral du moyen infrarouge (de 400 à 4 000 cm^{-1}) peut être, en particulier, utilisé pour caractériser la structure secondaire des protéines. Quand on soumet une molécule à une radiation infrarouge, sa structure moléculaire se met à vibrer. Ceci a pour effet de modifier les distances interatomiques (vibrations de valence ou d'élongation) et les angles de valence (vibrations de déformation). Lorsque la fréquence de l'oscillation d'une liaison atomique est égale à celle de l'onde émise à partir de la source, les liaisons moléculaires absorbent cette énergie ce qui conduit à l'apparition de bandes spectrales. Les régions caractéristiques des liaisons peptidiques sont les bandes amides I et II. La bande amide I (entre 1630 et 1660 cm^{-1}) reflète la vibration des liaisons C=O, et la bande amide II (entre 1520 et 1550 cm^{-1}) la vibration des liaisons C-H. L'analyse fine de la position et de la structure de ces bandes donne des informations sur la structure secondaire de la protéine étudiée ainsi que sur les différentes conformations secondaires de type hélice α, feuillet β, coude β ou pelote.

Mise en œuvre

L'appareil de mesure utilisé était un spectromètre-IRTF Bruker Tensor 27 (Bruker Optics, Marne La Vallée, France) équipé d'un détecteur telluride mercure/cadmium refroidi avec de l'azote liquide en mode réflexion totale atténuée (Attenuated Total Reflectance, ATR). Les suspensions de complexes ont été déposées sur un cristal Ge du support MIRacle (Pike Technologies, Madison, WI, USA). Les spectres ont été enregistrés dans la région 850-4000 cm^{-1} avec une résolution de 4 cm^{-1} auxquels a été soustrait le spectre de l'eau. Les spectres ont ensuite été tronqués à 1481-1718 cm^{-1} et normalisés avec un correcteur de signal multiplicatif (Martens et al., 2003). Le traitement des spectres a été réalisé par une analyse en composantes principales (principal component analysis, PCA) avec le logiciel R-package (R 2.9.2. Foundation for Statistical Computing)

III.2.3. Caractérisation des propriétés de gélification acide des complexes thermo-induits dans des systèmes laitiers

III.2.3.1. Préparation des systèmes laitiers

Les suspensions de complexes ont été tout d'abord diluées à 30 g L^{-1} dans le MUF, puis la phase solvante des suspensions de complexes thermo-induits a été remplacée par la phase solvante du lait (MUF) par dialyse contre du lait UHT. Les boudins de dialyse (6-8 kDa) (~200 mL) ont été placés dans un grand volume de lait (5 L) avec 0.2 g kg^{-1} de NaN_3, sous agitation à 4°C pendant une nuit. Les concentrations protéiques des suspensions, à l'issue de la dialyse, ont été vérifiées par mesure de l'absorbance à 280 nm en utilisant le coefficient d'extinction massique de 1.46 L g^{-1} cm^{-1} pour le WPI.

La poudre de PPCN a été reconstituée dans le MUF à une concentration de 65 g kg^{-1} sous agitation à 40°C pendant 4 h avec 0.2 g kg^{-1} de NaN_3, puis stockée une nuit à 4°C.

La gélification de 2 types de systèmes a été suivie : les suspensions de complexes seuls, et les laits reconstitués. Ces derniers ont été acidifiés à 35°C ou à 25°C selon les conditions du Tableau 4. La dose de GDL adéquate a été ajoutée au mélange protéique préalablement équilibré à la température de mesure, et dispersée pendant 2 min. Cinquante g ou 5 g du mélange ont été placés dans la géométrie du rhéomètre, selon qu'il s'agissait respectivement de cylindres coaxiaux ou d'un cône/plan (voir §III.2.3.2).

Tableau 4 : Composition des mélanges et conditions d'acidifications

	Systèmes laitiers		
	Suspensions de complexes seuls	Lait reconstitué à 35°C	Lait reconstitué à 25°C
Concentration en protéines (g L^{-1})			
Suspension de complexes du MUF	20	10	10
PPCN reconstitué dans le MUF	-	40	40
Dose de GDL (g kg^{-1})	17	18	19
Température	35°C	35°C	25°C
Temps pour atteindre pH 4.5 (h)	5-6	5-6	12-15

III.2.3.2. Suivi des propriétés viscoélastiques au cours de la gélification des systèmes laitiers

Principe

Les mesures rhéologiques dynamiques en oscillations de faibles amplitudes permettent de caractériser en continu, et de façon non destructive, la formation du gel laitier.

Une contrainte contrôlée est appliquée au produit. La contrainte varie sinusoïdalement au cours du temps par rotation d'un cylindre ou d'un cône plongé dans le lait autour de sa position d'équilibre. En réponse au mouvement imposé de la géométrie, une déformation du matériau peut être mesurée. Il s'agit également d'une fonction sinusoïdale du temps déphasée d'un angle δ par rapport à la contrainte imposée. Pour un produit purement solide, l'angle de déphasage est nul, le produit se déformant immédiatement à l'application de la contrainte. Pour un produit purement liquide, l'angle de déphasage est maximal et égal à $\pi/2$. Pour les matériaux viscoélastiques, dont font partie la majorité des produits alimentaires, l'angle de déphasage δ est compris entre 0 et $\pi/2$.

On peut alors calculer le module viscoélastique G* (en Pa) qui se décompose en un module élastique ou conservatif $G' = G^*\cos\delta$ et un module visqueux ou dissipatif $G'' = G^*\sin\delta$. La tan δ, égale à G''/G' et ses composantes caractérisent les propriétés visqueuses et solides du matériau.

Mise en œuvre

Le suivi des propriétés viscoélastiques au cours de la gélification a été effectué grâce aux rhéomètres AR1000 ou AR2000 de la société TA Instrument (Saint Quentin en Yvelines, France) équipés respectivement d'une géométrie à cylindres coaxiaux en aluminium de d'entrefer 1,95 mm, contenant 50 g d'échantillon ; ou d'une géométrie cône/plan en acrylique de 6 cm de diamètre et de 3°59 d'angle, contenant 5 g d'échantillon.

Les modules G', G'' et la tan δ ont été mesurés au cours de la gélification, avec une fréquence de 0,1 % et 1 Hz. Les rhéomètres appliquent une contrainte sinusoïdale et mesurent la déformation. Il s'agit d'une très faible déformation, puisque dans le cas des cylindres coaxiaux le cylindre intérieur parcourt une distance d'environ 2 µm autour de la position centrale. Une électrode de pH Inlab 415 (Mettler-Toledo, Viroflay, France) plongeait soit dans

le mélange protéique/GDL contenu dans la géométrie à cylindres coaxiaux, ou soit dans un flacon en verre thermostaté à la température de gélification et contenant le mélange, dans le cas de la géométrie cône/plan. L'électrode de pH a été reliée à un pH-mètre enregistreur (Consort C931, Bioblock Avantec, France). Ce dispositif permet de mesurer le pH en continu afin de déterminer les mesures rhéologiques en fonction du pH. Une fine couche d'huile de paraffine est ajoutée à la surface des mélanges pour empêcher l'évaporation de l'eau.

III.2.3.3. Caractérisation des propriétés mécaniques des gels acides finaux

a) Mesure de la résistance des gels

La résistance des gels acides des laits reconstitués (Tableau 4 et §III.2.3.2) a été caractérisée pendant l'application d'une forte déformation. Les gels formés dans la géométrie des rhéomètres et parvenus à pH 4.5, sont soumis à une étape de balayage en contraintes par le biais d'oscillations d'amplitude croissante de 0 à 3.5 kPa. La contrainte limite (en Pa) est définie comme le point où le G' commence à diminuer (rupture) et caractérise la résistance des gels. La contrainte limite a été déterminée à l'intersection entre les régressions linéaires du domaine viscoélastique linéaire et du domaine au delà de la rupture.

b) Mesure de la fermeté

En parallèle des suivis de gélification des laits reconstitués, 5 flacons en plastiques de 28 mm de diamètre intérieur, et 70 mm de hauteur ont été remplis avec chacun 10 g de mélange lait/GDL selon les conditions du Tableau 4. Les flacons ont été fermés et immédiatement incubés dans une étuve régulée à la température de mesure. Lorsque le pH atteint 4.5, les flacons ont été stockés une nuit à 4°C.

La fermeté des gels a été évaluée par un test de pénétrométrie consistant à mesurer la résistance à l'enfoncement d'un cylindre dans un produit. La mesure a été réalisé avec une machine de traction compression universelle INSTRON-4501 (Norwood, MA, USA) munie d'un capteur de force de 10 N. Les flacons ouverts ont été positionnés sur le support, et un cylindre en plastique de diamètre extérieur de 11 mm est enfoncé de 10 mm dans le gel avec une vitesse d'enfoncement de 120 mm min^{-1}. La pente initiale de la force de résistance mesurée

en fonction de la profondeur d'enfoncement du cylindre (en N mm^{-1}) a été utilisée pour caractériser la fermeté des gels.

III.2.3.4. Caractérisation des propriétés structurales des gels acides de lait

Les laits reconstitués préparés selon les conditions du Tableau 4, ont été marqués en ajoutant 0.2 g kg^{-1} d'une solution de rhodamine B isothiocyanate (85 g L^{-1} de RITC dissoute dans du diméthylsulfoxyde). La RITC se fixe sur les fonctions NH_2 des protéines et permet donc de visualiser le réseau protéique sans modifier la cinétique de gélifications (Guyomarc'h et al., 2009a). Les protéines sont donc localisées par le marquage en rouge de la RITC, tandis que les zones noires (non marquées) sont définies comme étant les pores du gel, c'est-à-dire une zone contenant du lactosérum, exempt d'objets de taille supérieure à la résolution du microscope (1 µm).

Une goutte du mélange lait/GDL a été déposée sur une lame cuvette recouverte d'une lamelle fixée de manière étanche avec du vernis (Figure 19). La lame a été incubée retournée dans une étuve régulée à la température de mesure. L'observation a été réalisée à pH 4.5, avec un microscope confocal inversé à balayage laser TE2000-E équipé d'un système d'imagerie Nikon C1Si (Nikon, Champigny-sur-Marne, France). La source d'excitation était un laser hélium-néon qui émet à une longueur d'onde de 543 nm. Le gel a été observé au grossissement x 100 avec un objectif immergé dans l'huile sur un plan focal à 5 µm de profondeur dans le gel. La lumière émise a été enregistrée à 590 nm par le détecteur. Les images obtenues ont été traitées et enregistrées avec une résolution de 512 pixels x 512 pixels à une cadence d'acquisition de 10.56 µs par pixel, à l'aide d'un logiciel EZ-C1 (Nikon).

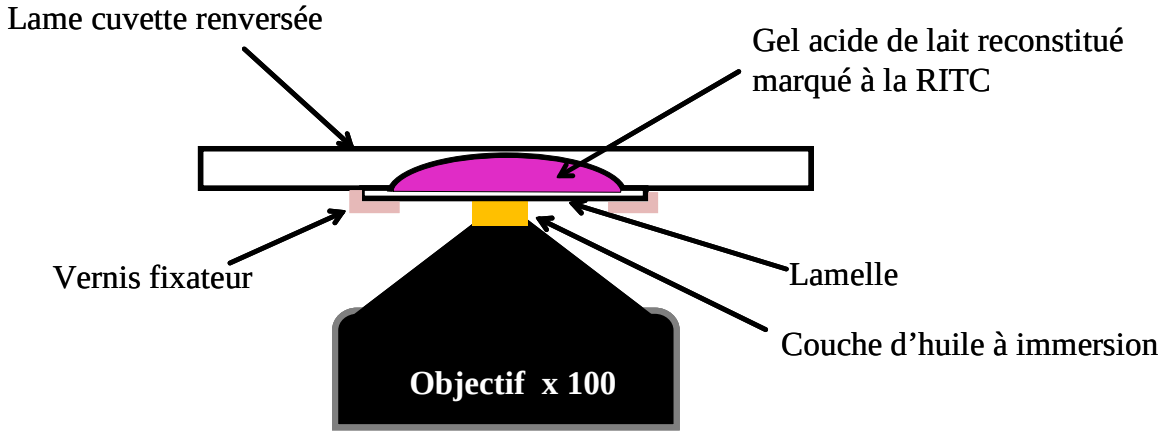

Figure 19 : Dispositif expérimental pour l'observation du gel acide de lait reconstitué, marqué à la rhodamine B isothiocyanate (RITC), au microscope confocal

Une égalisation de l'histogramme a été appliquée sur toutes les images pour enlever des variations d'intensité, et convertir l'image en niveaux de gris. Les zones blanches sont les protéines, et les zones noires les pores du gel. Ensuite, une méthode d'analyse granulométrique des niveaux de gris, par fermeture, a été réalisée comme décrite par Devaux et al. (2008). La méthode consiste à appliquer en se déplaçant sur toute l'image un carré et à faire disparaitre les objets noirs plus petits que ce carré, c'est-à-dire qu'ils deviennent blancs. En appliquant des carrés de taille croissante (de 0.75 à 50 µm), on obtient à chaque fois une nouvelle image dont on calcule la somme des niveaux de gris. En considérant la dérivée de la somme des niveaux de gris obtenus par rapport à la taille du carré, on obtient une distribution de taille des régions noires dans l'image, c'est-à-dire la distribution de la taille des pores du gel. La comparaison de toutes ces courbes de distribution a été réalisée par une analyse en PCA avec le logiciel R-package. L'analyse granulométrique par ouverture, qui permettrait d'évaluer la taille des objets protéiques, n'a pas pu être employée car la présence d'insolubles dans certains échantillons affectait la procédure.

Chapitre IV.

Résultats et discussion – Partie 1

Rôle de la caséine-κ dans les complexes thermo-induits sur l'établissement des interactions protéiques au cours de la gélification du lait

IV.1. Introduction

Ce premier chapitre de résultats concerne le rôle de la teneur en caséine-κ dans les complexes thermo-induits sur l'établissement des interactions protéiques au cours de la gélification acide. En effet, d'après notre étude bibliographique (§II.6.2.2), nous avons suggéré que la caséine-κ pourrait favoriser l'établissement des interactions entre les particules au cours de la gélification, c'est à dire entre les micelles de caséines et les complexes, tous contenant de la caséine-κ. En modifiant la proportion en caséine-κ des complexes thermo-induits, nous testons ici l'hypothèse du rôle de médiateur de cette caséine dans l'association complexes/micelle au cours de l'acidification. Selon la stratégie adoptée tout au long du projet, les propriétés physico-chimiques de surface des complexes thermo-induits ont été caractérisées afin d'établir un lien entre les propriétés des complexes et la fonctionnalité de gélification acide des laits modèles.

Cette première partie de résultats a fait l'objet d'une conférence lors d'un symposium de la fédération internationale de laiterie (IDF) à Tromso en Norvège en juin 2010 (« IDF Symposium on Microstructure of Dairy Product »). Un article a récemment été publié dans le journal *International Dairy Journal*, dans un volume spécial consacré à ce symposium. L'intégralité de l'article est proposée dans ce chapitre pour présenter les résultats obtenus et leur discussion.

IV.2. Article 2

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On how κ-casein affects the interactions between the heat-induced whey protein/κ-casein complexes and the casein micelles during the acid gelation of skim milk

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Abstract - Heat treatment of milk at 85-95°C has long been reported to increase the pH of gelation and firmness of acid milk gels; hence its wide application in yoghurt manufacture. These changes have been attributed to the formation of heat-induced whey protein/κ-casein complexes in the milk, to which heat-denatured whey protein ingredients may be substituted. However, variations in resulting gels show that a possible role of κ-casein in determining the functional acid-gelation property of the complexes needs investigating. Model heat-induced whey protein/κ-casein complexes were produced of κ-casein content from 0 to 40% (w/w), but of similar size, secondary structure, surface hydrophobicity and thiol/disulphide distribution. These complexes were added to whey protein-free skim milk systems and the resulting acid-gelation behaviour of the milks was evaluated. The results showed a modification of the pH of gelation that was explained more by variation of the pI of complexes than by the κ-casein content.

1. Introduction

The application of extensive heat treatment to milk in order to increase the firmness of yoghurt gels has long been exploited industrially. Since the work of, e.g., Dannenberg & Kessler (1988b), Lucey et al. (1997a) or Parnell-Clunies et al. (1986b), the increase in the textural properties of acid milk gels with prolonged heating has been unambiguously attributed to the denaturation of the whey proteins and their aggregation into heat-induced complexes.

Various studies have shown that the presence of complexes in heated milk increases the pH at which gelation starts and yields stranded, connected and porous gel microstructures, which also have higher elastic modulus and water retention capacity when compared to those of unheated milk (see review by Donato & Guyomarc'h (2009). Similar results have been obtained when adding heat-aggregated whey proteins to milk (Janhøj & Ipsen, 2006; Schorsch et al., 2001; Vasbinder et al., 2004).

However, the heat-induced complexes that are formed when heating milk deeply differ from whey protein particles in the sense that they also involve casein molecules, especially the cysteine-containing κ-casein (Anema, 2007; Donato & Dalgleish, 2006; Jean et al., 2006; Renan et al., 2006; Singh & Creamer, 1991). Interestingly, other studies have proposed that the interaction between the heat-induced complexes and the casein micelle was essential to the building of the protein network (Kalab et al., 1976; Lucey et al., 1998b) and it has been demonstrated that the two types of particles are co-located in the final gel (Vasbinder et al., 2004). Interaction between the complexes and the casein micelles can occur during heating to form micelle-bound complexes (Anema, 2007; Corredig & Dalgleish, 1996; Jang & Swaisgood, 1990; Lucey et al., 1998b), but it probably also occurs between the serum complexes and the casein micelles, early in the course of acidification (Alexander & Dalgleish, 2005; Guyomarc'h et al., 2009a).

When heat-aggregated whey protein ingredients are added to skim milk, the heat treatment of the mixture has been shown to further increase the final firmness and homogeneity of the resulting acid gel (Kalab et al., 1976; O'Kennedy & Kelly, 2000; Schorsch et al., 2001). Schorsch et al. (2001) suggested that the added whey protein complexes interacted with κ-casein on the surface of the casein micelles on heat treatment, thus favouring connectivity of the acid gel.

The κ-casein seems to play a role in determining the acid-gelation functionality of the heat-induced whey protein complexes. However, this has not yet been evaluated. Considering the location of the κ-casein on the surface of the casein micelle and its specific role as the micelles' natural stabiliser (Dalgleish, 1998; Tuinier & de Kruif, 2002; Walstra, 1990), one could propose that κ-casein helped acid gelation either through depleting the casein micelle of its stabiliser when involved into serum heat-induced whey protein/κ-casein complexes or

through mediating the association of the complexes with the casein micelles, possibly as an anchor between the two kinds of particles. In particular, serum complexes that contain κ-casein may bind to the casein micelle through the return of the casein to its natural location.

To investigate this latter proposition, model heat-induced whey protein/κ-casein complexes were produced, which proportion of κ-casein varied from 0 to 40% (w/w), all other properties being reasonably equal. As complexes were produced in a different way than during the heat treatment applied to pre-heated milk (e.g., 90 °C, 10 min), a deep characterisation of their surface properties was performed to check that they are not too different from the heat-induced complexes of milk. These complexes were introduced to whey protein-free skim milk systems and the resulting acid-gelation behaviour of the milks was evaluated.

2. Materials and Methods

2.1 Materials

Native micellar casein (NMC - 870 g of proteins on dry basis - whey protein level: 5%) was prepared as described by Schuck et al. (1994). Briefly, raw milk was skimmed at ~50 °C then microfiltered through 0.1 µm cut-off ceramic membrane, diafiltered, evapo-concentrated and spray-dried. The microfiltration permeate, containing the native whey protein, was concentrated by ultrafiltration onto 8 kDa ceramic membrane, diafiltered and freeze-dried to yield a native whey protein isolate (WPI - 970 g of proteins on dry basis - whey protein level: 80%). The milk ultrafiltration permeate (MUF) resulting from UF-concentration of the whey protein fraction was collected and stored at 5 °C after addition of 0.2 g L^{-1} sodium azide.

The κ-casein was extracted from acid casein by a method adapted from Zittle & Custer (1963). After dispersion in urea, the different caseins were selectively precipitated by addition of solid ammonium sulphate at various pH values, extensively dialysed then freeze-dried (κ-casein - 980 g of proteins on dry basis - whey protein level: 5%).

All other reagents were of analytical grade.

2.2. Preparation of the heat-induced complexes

Mixtures of 15–20 g kg^{-1} WPI and 0–10 g kg^{-1} (0-40%, w/w) of κ-casein were prepared in 0.1 M NaCl, 0.2 g L^{-1} NaN_3, pH 7.0 (Guyomarc'h et al., 2009b).

Compositions were checked using absorbance at 280 nm and extinction coefficient values of 1.046 and 0.930 L g^{-1} cm^{-1} for the WPI and the κ-casein, respectively. The aqueous solvent and the WPI solution at 15-20 g kg^{-1} were sequentially used as blanks. Calculation indicated that the corresponding weight proportion of κ-casein in the mixtures varied from 0 to 40% (w/w) of the total protein present. The mixtures were then heat-treated in tightly sealed vials of 10 mL at 80 °C for 24 h in a water bath, and subsequently cooled in running tap water. According to the literature (Baussay et al., 2004; Le Bon et al., 1999; Mahmoudi et al., 2007; Zuniga et al., 2010), the maximum conversion of whey proteins in complexes needs 24 h of heating at 80 °C in similar conditions. Obviously, the maximal denaturation of the whey proteins is reached in less than 1 h (Croguennec et al., 2004; Donato et al., 2009; Zuniga et al., 2010), but the formation of complexes is a much longer process. When heating pure β-lactoglobulin at 80 °C under similar conditions, Le Bon et al. (1999), Mahmoudi et al. (2007) and Baussay et al. (2004) have demonstrated that heating times of up to 24 h were necessary to ensure the maximal conversion of whey proteins into heat-induced complexes and that complexes do not grow anymore. A heat treatment at 80 °C during 24 h was therefore performed to ensure a maximal content in heat-induced complexes and a lower content in residual non-aggregated species.

We only mention here that isolation of the resulting heat-induced complexes by size exclusion chromatography (SEC) and reverse-phase high performance liquid chromatography (RP-HPLC) analysis of their composition confirmed that not all κ-casein was incorporated into the complexes as opposed to ~ 96% of the initial supply in whey proteins, thus yielding an actual proportion of 0-25% (w/w) κ-casein in the complexes. These results clearly confirmed earlier suggestions that not all the κ-casein is able to form heat-induced whey protein/κ-casein complexes (Gerrard & Brown, 2002; Singh, 1991; Walstra et al., 1999). The near 100% conversion of the limiting reactant (the whey proteins) as evidenced by RP-HPLC after incubation of the samples in urea and β-mercaptomethanol also indicated that no covalent

bonds other than disulfide ones were formed during heating in the present conditions. A control whey protein complex (WPIA) was also prepared as described by Vasbinder et al (2003a). Briefly, a 90 g kg^{-1} solution of WPI in deionised water with 0.2 g kg^{-1} NaN_3 was adjusted at pH 7.5 then heated at 68.5 °C for 2 h. These conditions yield complexes having comparable size to that of the serum complexes of milk.

2.3. Determination of the size of the complexes

2.3.1. Dynamic Light Scattering

Particle size analysis was performed on the WPI/κ-casein heated mixtures using dynamic light scattering (DLS) at a fixed angle of 173° on a Zetasizer nano ZS (Malvern Instruments, Orsay, France). The laser was a He–Ne laser, with λ=633 nm. Samples were equilibrated at 20 °C, diluted in deionised water to meet the Zetasizer operating range, and allowed to stand at 20 °C for a standardised period of 20 min to ensure proper equilibration of the diluted system prior to analysis. The refractive index of water was 1.330, and the viscosity of water was 1.0031 mPa s at 20 °C. The presented results are the average of 10 readings, and each sample was analysed in triplicate. The data were computerised using a CONTIN modelling routine and the relaxation time distribution was transformed into a distribution of hydrodynamic diameters, D_h, using the Stokes–Einstein relationship: $D = kT/(3\pi\eta D_h)$ where k is the Boltzman's constant and η is the viscosity of water at 20 °C. Due to the complexity of the analysed mixtures, which may contain e.g. non-reacted κ-casein (see section 2.2), the output distributions sometimes showed multiple modes. For that reason, the mode of the major population (the whey protein/κ-casein complexes), and not the calculated Z average value, was taken as the average D_h of the complexes.

2.3.2. Size exclusion chromatography coupled with online multi-angle laser light scattering

Size Exclusion Chromatography (SEC) analysis of the heated WPI/κ-casein mixtures was performed on a Sephacryl S-500 Hi-Prep 16/90 column (Amersham Biosciences, Orsay,

France) at room temperature in isocratic conditions using 0.1 M Tris, 0.5 M NaCl and 10 mM NaN_3, pH 7 as the mobile phase, filtered through 0.1 µm and degassed. Loop size was 100 µL, flow-rate was 0.5 mL min^{-1} and absorbance was at 280 nm. The chromatographic system was connected downstream to a 18-angle DAWN-Heleos II Multi-Angle Laser Light Scattering (MALLS) detector equipped with a K5 cell and a 658-nm laser diode (Wyatt Technology, Santa Barbara, CA, USA) and to an Optilab rEX refractometer operating at 25 °C with a 685-nm laser diode (Wyatt Technology). Data were collected and analysed using an Astra software version 5.3.4.11 (Wyatt Technology); the molecular weight (M_w) and the radius of gyration (R_g) were determined online using a value of the refractive index increment, dn/dC, of 0.183 mL g^{-1} and the Zimm method. In order to compare samples, the values of the M_w and of the R_g found at the median retention time of the complexes' peak, i.e., at 50% of the total peak area, were considered (Figure 20).

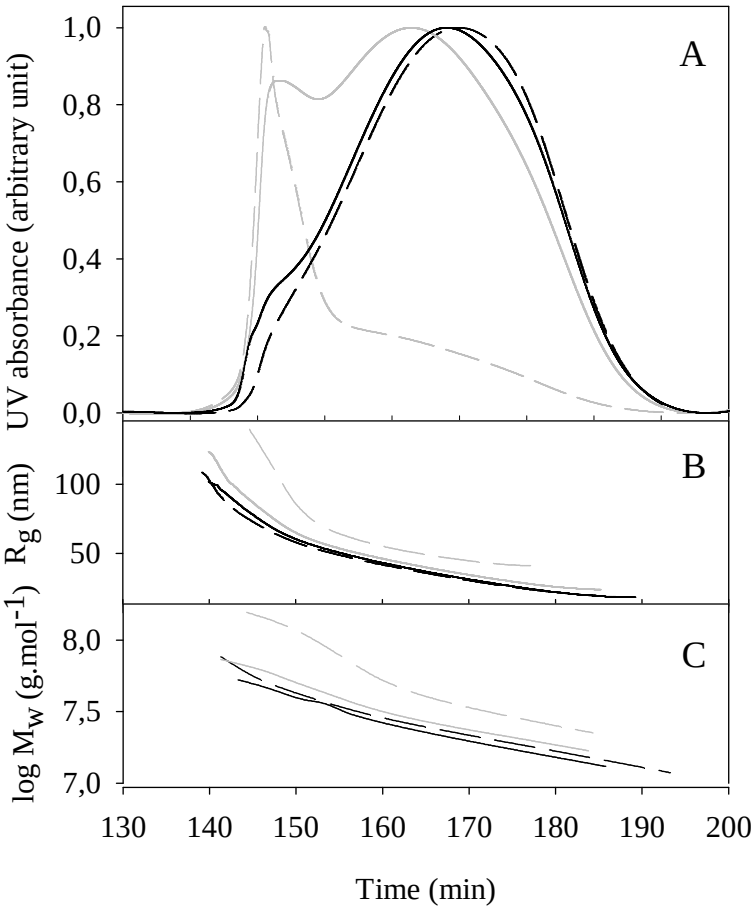

Figure 20 : Typical size exclusion chromatography absorbance profiles at 280 nm (A), coupled with multi-angle static light scattering online calculations of the radius of gyration (R_g) (B) and of the logarithm of the molecular weight (log $M_{w)}$ (C) of the eluting particles of mixtures of whey protein isolate (WPI) and κ-casein, heated at 80 °C for 24 h in 0.1 M NaCl, pH 7.0. Selected profiles of mixtures with increasing % (w/w) κ-casein are shown for clarity: grey dotted line = 15.8%, grey line = 19.5%; black dotted line = 28.6%; black line = 40.0%.

2.4. Surface hydrophobicity

The surface hydrophobicity of the heat-induced complexes was estimated using the 8-anilino-1-naphthalene sulphonic acid (ANS)-binding fluorimetric assay, using a LS50B spectrophotometer (Perkin Elmer, Saint Quentin-en-Yvelines, France). To a constant protein concentration of complexes diluted in a maleate-NaOH buffer at pH 6.7, 0-40 µM of ANS were added so that the ANS:protein ratio was 0, 1, 2, 3, 6 or 10 mol g^{-1} and that absorbance at 390 nm did not exceed 0.1-0.15 as the upper limit of the linear range for fluorescence emission by ANS-β-lactoglobulin (β-lg) complexes. Excitation wavelength was 390 nm and emission was measured at 480 nm. The emission and excitation slits were both set at a bandwidth of 2.5 nm. The increase in fluorescence (F) with increasing ANS (in µM) addition was modelled using the Michaelis-Menten equation (Bonomi et al., 1988):

$$F = F_{max} \times [ANS] / (K_d + [ANS]) \quad \text{(Équation 7)}$$

where F_{max} is the maximum fluorescence at the saturating ANS concentration and relates to the number of surface hydrophobic sites of the protein. K_d is the ANS concentration at $F_{max}/2$ as a result of Michaelis-Menten kinetics and is defined as the dissociation constant of the fluorescent ANS-protein complex. The protein surface hydrophobicity can eventually be described through calculating the protein surface hydrophobicity index (PSH, in µmol L^{-1}ANS x µg L^{-1} prot) as previously described (Erdem, 2000):

$$PSH = Fmax/(Kd[protein]) \quad \text{(Équation 8)}$$

2.5. Apparent isoelectric point (pI)

The apparent isoelectric point (pI) of the heat-induced whey protein/κ-casein complexes was determined as in Jean et al. (2006) or Guyomarc'h et al. (2007b) using interpolation to 0 µm cm V^{-1} s^{-1} of the experimental measurement of the electrophoretic mobility of the complex (mb, in µm cm V^{-1} s^{-1}) as the pH of the medium is varied from ~2 to 6.7. The medium used for dilution and measurement was MUF, adjusted at 6 different pH values using 5 M HCl and to which calcium ions were added in the form of $CaCl_2$ as to reach the levels found in skim milk acidified to the same pH values (Mekmene et al., 2009). Preliminary experiments have indeed validated that the mb profile of control WPIA measured across pH in the MUF-$CaCl_2$ series

was superimposable to that of WPIA measured in the ultrafiltration permeates of heated skim milk adjusted at the same pH values (results not shown). Mb was measured at 50 V on the same Zetasizer nano ZS as for DLS measurements. The samples equilibrated at 20 °C were diluted in the MUF-$CaCl_2$ media at 20 °C and left at that temperature for 20 min to ensure proper mineral equilibrium.

2.6. Determination of surface and free sulfhydryl groups and of total cysteine

Three different types of S groups could be assayed using the 5,5'-dithio-bis-2-nitrobenzoïc acid (DTNB) (Ellman, 1959): the accessible thiol groups of the complexes (surface SH), the total free thiol groups (SH) and the total cysteine residues (S).

Surface SH were determined on aliquots of the heated WPI/κ-casein mixtures diluted at ~ 5 g kg^{-1} in a non-dissociating buffer (0.2 M Tris-HCl, pH 7.4). 1 mL of DTNB (1 g.L^{-1}) was added to 9 mL of the diluted samples and reacted with the thiol groups to release 2-nitro-5-thiobenzoate anion (TNB^{2-}) in the aqueous phase. After incubation at room temperature for 5 min, ammonium sulphate (0.6 g mL^{-1}) was added to the solution in order to precipitate the proteins and remove them using filtration through Whatman 40 paper. The TNB^{2-} product present in the clear filtrate can be assayed using absorption at 412 nm on a spectrophotometer (Uvikon 922, Kontron France, Paris, France).

Total free SH were determined on aliquots of the same samples, diluted at ~2 g kg^{-1} in a dissociating buffer (8 M urea, 0.05 M citrate tri-sodium, 5 g L^{-1} sodium dodecyl sulphate, 0.2 M Tris, pH 7.4). 35 µL of DTNB at 1 g L^{-1} were added to 1 mL of the diluted samples, that were further prepared as above described.

Total cysteine residues were also determined on 100 µL aliquots of the WPI/κ-casein mixtures, to which 20 µL mL^{-1} of β-mercaptoethanol and 1 mL per mL of urea 10 M were added. After 1 h incubation at 37 °C, to eliminate excess β-mercaptoethanol, the proteins were precipitated using 12% (v/v) trichloroacetic acid (TCA) and collected using centrifugation (5000 x g, 10 min, 20 °C). The collected proteins were then dissolved in the above dissociating buffer, after what the same preparation method was used.

The analyses were performed 2 times and results were expressed in µmol per g of protein using a molar extinction coefficient determined for each buffer using calibration with cysteine solutions ranging from 0 to 0.028 mM.

The content in total cysteine residues was also determined as previously described by Croguennec et al. (2001). Briefly, solutions of native WPI and casein-free complexes were first oxidised with performic acid according to Moore (1963), then acid-hydrolysed at 110 °C for 24 h and determined by chromatography (Spackman et al., 1958). The analyses were performed twice.

Experimental values for total S groups were compared to theoretical values using theoretical protein concentrations in whey and protein composition from Walstra & Jenness (1984b). With whey containing 7 g kg^{-1} proteins, amongst which 3.2 g kg^{-1} β-lg, 1.2 g kg^{-1} α-lactalbumin (α-lac) and 0.4 g kg^{-1} bovine serum albumin (BSA) and 5, 8, 35 and 2 cysteine residues in β-lg, α-lac, BSA and κ-casein, respectively, the theoretical content in cysteine was estimated.

2.7. Secondary structure of proteins

The secondary structure of proteins was assessed by Fourier Transform Infra-Red measurements (FTIR) using a Bruker Tensor 27 instrument (Bruker Optics, Marne La Vallée, France) equipped with a mercury/cadmium telluride detector cooled with liquid nitrogen in the Attenuated Total Reflectance (ATR) mode using a single-reflection Ge crystal in a MIRacle accessory (Pike Technologies, Madison, WI, USA). Spectra were recorded in the region 850-4000 cm^{-1} at a 4 cm^{-1} resolution at room temperature taking the spectrum of water as the background. Each WPI/κ-casein mixture, either heated or not, was measured at least 6 times with a careful cleaning of the crystal between 2 measurements. Spectra were truncated to 1481-1718 cm^{-1} and normalised with the extended multiplicative scatter correction (Martens et al., 2003). The terms used in the regression were the mean spectrum, the liquid water spectrum and linear and quadratic functions of the wave number. This procedure allows correcting each spectrum for variability in overall intensity, variability due to the water signal (hence, to total protein concentration) and to the baseline changes. A principal component analysis (PCA) was performed on spectra from unheated and heated protein suspensions. PCA was also applied on

heated suspensions solely. Statistical analyses were done using the R package (R 2.9.2. Foundation for Statistical Computing) as an open source statistical computing environment.

2.8. Acid-gelation behaviours of the reconstituted milk system

First, the whey protein/κ-casein complexes were extensively dialysed (6-8 kDa, Medicell International Ltd., London, UK) at 4 °C against commercial UHT half-fat milk in order to replace their solvent phase with milk permeate. Their final concentration was determined by absorbance measurements at 280 nm after dissociation in urea and dithiothreitol (DTT) and calibration with WPI. A mother suspension of NMC was reconstituted at 65 g kg^{-1} in MUF at 40 °C. The NMC suspension, the whey protein/κ-casein complexes and MUF were eventually mixed as to reach a total protein content of 50 g kg^{-1} and a NMC to complexes ratio of 80:20 (w/w).

Formation of the acid gels was monitored by measuring the elastic modulus (G') and loss tangent (tanδ), defined as the ratio of the viscous to the elastic modulus of a system, G"/G' of the reconstituted milk systems on acidification with 18 g kg^{-1} glucono-δ-lactone (GDL) at 35 °C using an AR1000 rheometer (TA Instruments, Guyancourt, France) equipped with a coaxial geometry and using the oscillatory mode with coaxial cylinder geometry at a frequency of 1 Hz and a strain of 0.01. The pH was simultaneously recorded in-situ and with time using an Inlab 415 electrode (Mettler-Toledo, Viroflay, France) placed in the rheometer geometry (Consor C931, Bioblock Avantec, France). NMC-complexes in MUF mixes were placed in a water bath at 35 °C for 10 min after addition of 0.2 g kg-^{1} rhodamine B isothiocyanate solution (4 g L^{-1} RITC in dimethylsulfoxyde, Sigma-Aldrich, St Quentin Fallavier, France). GDL was dispersed 2 min at 35 °C and the suspension was immediately placed in the cup and covered with a thin layer of paraffin to prevent dehydration during incubation. Gelation was defined as the moment when G' > 1 Pa and tanδ < 1. Preparation of each reconstituted milk system was performed twice, and acid gelation was monitored on at least 2 aliquots of each reconstituted system.

2.9. Microstructure of the acid gels

Immediately after GDL dispersion, one drop of the RITC-labelled milk system was laid on a conclave slide, covered by a cover slip sealed with nail varnish, and incubated at 35 °C. When the pH reached 4.5, the slide was introduced to a TE2000-E inverted confocal laser scanning microscope (CLSM) equipped with a Nikon C1Si imaging system (Nikon, Champigny-sur-Marne, France) with helium-neon lasers (laser) emitting 543 nm. A Nikon 100x oil-immersion objective with 1.40 numerical aperture was used. The emitted light was recorded at 590 ± 25 nm. The software used for image acquisition was EZ-C1, version 3.40 (Nikon). Digital images were recorded at 15 μm depth into the sample in 512 pixels x 512 pixels resolution and at 10.56 μs pixel dwell. Pinhole diameter and all other settings were also kept constant. Image brightness was auto-adjusted using the software EZ-C1 FreeViewer, version 3.20 (Nikon).

3. Results and discussion

3.1. Physicochemical properties of the heat-induced complexes

It is now well known that casein molecules have an inhibitory effect on the heat-induced aggregation of the globular proteins, including whey proteins (Yong & Foegeding, 2010). Changes in the size of the present heat-induced whey protein/κ-casein complexes as a function of the proportion of κ-casein were therefore evaluated through measurement of their hydrodynamic radius, R_h, using DLS; of their molecular weight, M_w, and of their radius of gyration, R_g, using SEC-MALLS (Figure 21). In agreement with previous reports using the same separation column, the heat-induced whey protein/κ-casein complexes eluted in a broad peak ranging between exclusion and 260 min (Figure 20) (Donato & Dalgleish, 2006; Renan et al., 2007). The values of M_w and R_g found at the maximum UV absorbance of the pic of the complexes was taken as representatives of the whole distribution, and plotted in Figure 21B and C as a function of the proportion of κ-casein in the mixtures.

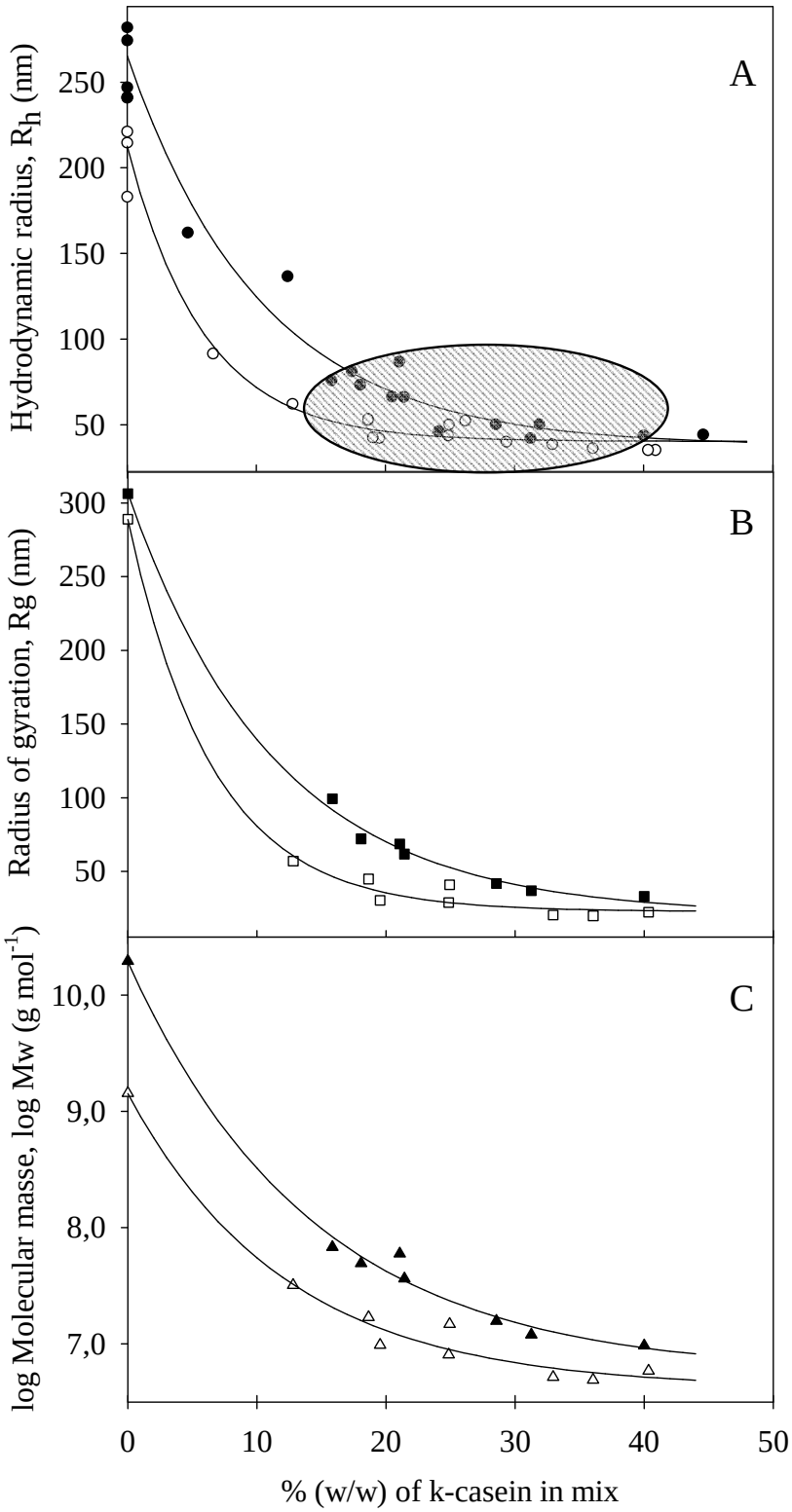

Figure 21 : Hydrodynamic radius measured by DLS (R_h) (A) of the whey protein/κ-casein complexes. Radius of gyration (R_g) (B) and logarithm of the molecular weight (log M_w) (C) measured taken at the mode of the size exclusion peak of the whey protein/κ-casein complexes, as a function of the % (w/w) of κ-casein for 15 (open symbols) or 20 g kg^{-1} (closed symbols) of WPI present, heated at 80 °C for 24 h in 0.1 M NaCl, pH 7.0

In agreement with Guyomarc'h et al. (2009b), the R_h of the complexes decreased exponentially from over 200 nm to below ~50 nm as more κ-casein was present (Figure 21A). At low κ-casein concentrations, the size of the complexes increased with increasing WPI concentration, but as little as 5% (w/w) κ-casein addition induced a 2-fold reduction in the R_h

of the complexes. On the other hand, the R_h tended towards a plateau value of ~40 nm at either WPI concentrations as soon as 30% or more κ-casein was involved. The growth of the heat-induced complexes therefore appeared clearly inhibited by κ-casein, as modelled by the following equation:

$$R_h = S1 \times \exp(-\kappa\text{-casein}/\tau) + S2 \qquad \text{(Équation 9)}$$

where S2 represents the size of the κ-casein-rich complexes, (S1+S2) represents the size of κ-casein-free complexes and depended on the initial concentration of WPI (S1 ≈ 165 or 220 nm at 15 or 20 g L^{-1} WPI, respectively) and τ, the curvature, gives the intensity of the inhibitory effect of κ-casein addition onto the growth of complexes (τ ≈ 9 or 13 at 15 or 20 g L^{-1} WPI, respectively). The model was found to fit other data (Guyomarc'h et al., 2009b) with agreeing outputs (not shown).

As expected, Figure 21B and C also showed that the κ-casein content in the WPI/κ-casein mixtures adversely affected R_g and M_w of the resulting heat-induced complexes. However, cut-off of the SEC column at M_w values of ~10^8 g mol^{-1} prevented sensitive discrimination of the lower κ-casein proportion range. Maximum M_w and R_g values were found in 15 and 20 g L^{-1} WPI solutions, respectively over ~10^9 g mol^{-1} and 250 nm, and tended towards constant values of 5.10^6 g mol^{-1} and ~30 nm as the concentration of κ-casein increased. R_g and $\log(M_w)$ can be adjusted with the same model (eq. 3) as a function of the κ-casein content.

Interestingly, the curvature parameter, τ, seemed to depend on the concentration of WPI, as it was consistently higher at 20 g L^{-1}. This suggested that the heat-induced complex formation was essentially driven by the whey protein fraction, while κ-casein rather acted as an inhibitor of the propagation. This suggestion was in agreement with earlier views that proposed the κ-casein as a dead-end in the reaction, either as a chaperone of the heat-activated whey proteins or as terminating thiol/disulphide exchanges (Donato & Guyomarc'h, 2009). The results therefore confirmed that the size of the heat-induced whey protein/κ-casein complexes was affected by the proportion of κ-casein incorporated. However, previous reports have suggested that larger complexes formed at pH 6.7 with higher whey protein to casein ratio yielded firmer acid gels (Guyomarc'h et al., 2009a; Puvanenthiran et al., 2002). To control this bias, it was

decided to study only WPI/κ-casein complexes of maximum 100 nm diameter, i.e., having 15 to 40% (w/w) κ-casein (Figure 21: hatched zone). When necessary, a κ-casein-free, ~100 nm diameter WPIA complex was used to extend this range to 0-40% κ-casein.

Other physicochemical properties that are also susceptible to vary with composition of the complexes and to affect the course of gelation of the milk systems have further been tested. It was found that the pI value of the various complexes decreased linearly and significantly ($P<0.05$) from ~4.6 to ~4.3 as the proportion of κ-casein increased from 0 to 40%, whatever the WPI concentration (Figure 22B). This effect could be attributable to the exposure of the κ-casein to the solvent, of which 55-60% bears acidic glycosyl side chains (Saito & Itoh, 1992; Vreeman et al., 1986). Indeed, the pI of pure κ-casein unheated and heated (68.5 °C for 2 h) was measured and found to be 4.0, in contrast to pure non-heated (pI 5.0) or heated WPI (pI 4.9).

In contrast, the surface hydrophobicity of the complexes showed to be consistent across composition. The PSH index was 0.10 ± 0.02 at all WPI and κ-casein concentrations. This value was smaller that the PSH values reported for casein micelles by Iametti et al. (1993) after isolation from heated skim milk, and sensibly lower than that of heated whey protein concentrates as reported by Moro et al. (2001). The differences probably arose from variation in the calculation of the PSH index, as well as from the low protein and ANS concentrations used in the present study to keep below ANS quenching.

Total cysteine contents obtained by Ellman's reaction and by chromatographic analysis of amino acids were 155 ± 48 and 178 ± 12 µmol g^{-1} protein, respectively. The lower value in the Ellman's method could arise from an oxidation reaction during the determination, as only the SH groups were measured in this method, while in the other method, the amino acids were first oxidised by performic acid. Furthermore, these values were slightly lower than the theoretical estimated value (252 µmol g^{-1}). This discrepancy could be due to the composition of whey and the process used to the preparation of the WPI powder.

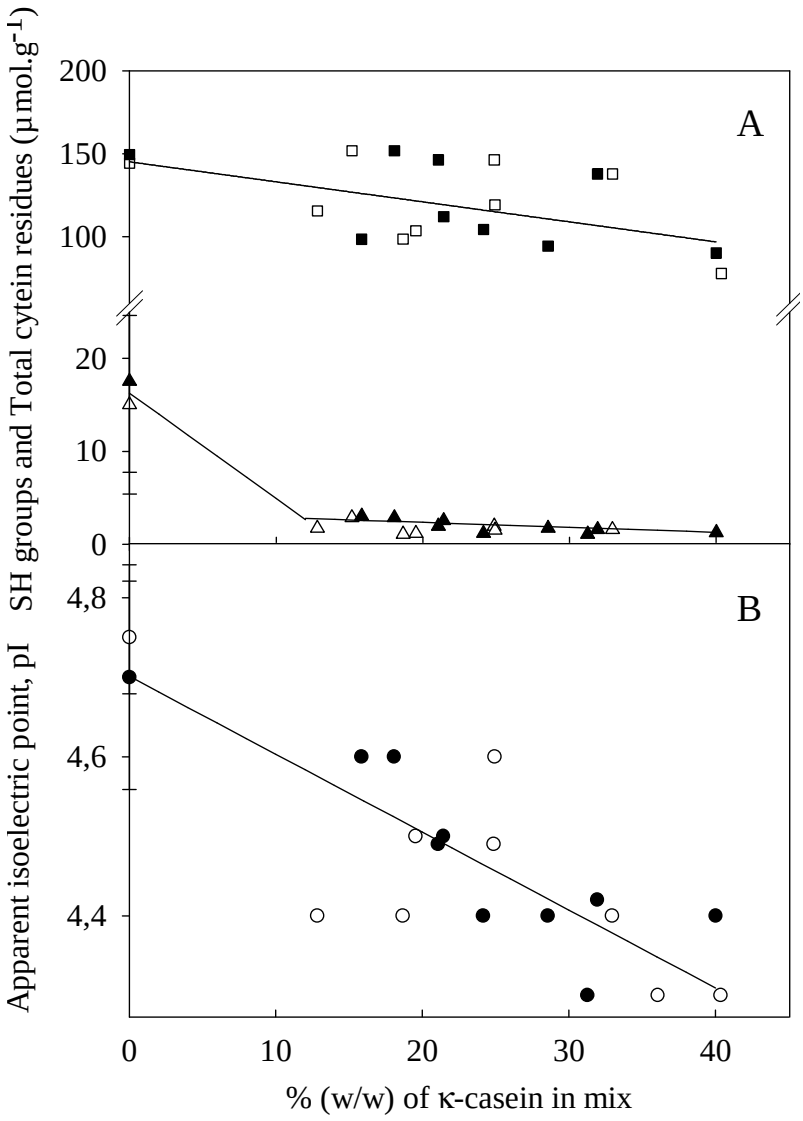

Figure 22 : SH groups (A, triangles), total cysteine residues (A, squares) and Apparent isoelectric point (pI) (B) assayed in heated (80 °C for 24 h) mixtures of 15 (open symbols) or 20 g kg^{-1} (closed symbols) WPI and κ-casein in 0.1 M NaCl, pH 7.0, as a function of the % (w/w) of κ-casein present

Figure 22 showed that the total cysteine content S in the complexes seemed to decrease from ~150 to ~100 µmol g^{-1} total protein as κ-casein increased from 0 to 40% (w/w) in the mixtures, most likely because κ-casein only contains 2 cysteine residues per mole while the whey protein generally contain larger number of this amino acid. However, the effect was not significant (P>0.1). The total free thiol content SH followed the same pattern as the S content, i.e., decreased very gently from ~3.0 to ~1.5 µmol g^{-1} with increasing % of κ-casein (P>0.1), except in κ-casein-free complexes where values of 10-20 µmol g^{-1} could be recorded. These results suggested that the κ-casein reduced the number of free thiol groups available for thiol/disulfide exchanges, which could account for its inhibitory effect on the growth of the heat-induced complexes (Figure 22). Possibly, κ-casein is more prone to oxidation into disulfide bonds than to propagating thiol/disulfide exchanges (Owusu-Apenten & Chee, 2004).

Surface SH groups were also assayed in mixtures heated at 80 °C for 24 h but yielded negligible values (not shown), despite the presence of SH groups in κ-casein-free complexes. Possibly, exposure of SH groups to the solvent and other reactant proteins decreases with heating time (Alting et al., 2002), thus contributing to finite growth of the whey protein complexes.

Secondary structures of the unheated and heat-treated protein mixtures were studied by FTIR and PCA. PC1, PC2 and PC3 explained 62.3, 15.8 and 8.8% of variance, respectively. Figure 23A shows that the unheated and heated samples were well separated into 2 groups, the scores on PC2 seemed to decrease as the proportion of κ-casein in the original mixtures increased. The spectral pattern of PC1 (Figure 23A) showed negative peaks at 1659, 1634 and 1587 and a sharp positive peak at 1616 cm^{-1} and smaller ones at 1639 and 1535 cm^{-1}. Increasing the scores on PC1 with heating means mainly a decrease in the intensity of the peaks at 1659 and 1587 cm^{-1} and an increase in intensity of the peak at 1616 cm^{-1}, leading to a broadening of the amide I band towards lower frequencies. The PC2 shows mainly a band at 1634 cm^{-1}. The decrease in scores on PC2 with the increase in κ-casein proportion in the mixtures means a smaller band at 1634 cm^{-1}. It is well known that the amide I band (1600-1700 cm^{-1}) is very sensitive to changes in the secondary structure of proteins. From previous studies on the denaturation/aggregation of proteins, the band at 1659, 1634 and 1616 cm^{-1} were assigned to α-helix + disordered structures, to intramolecular β-sheets and to intermolecular antiparallel β-sheets associated with protein aggregation, respectively (see papers on β-lg: (Gosal et al., 2004; Kehoe et al., 2008; Oboroceanu et al., 2010; Sanchez & Fremont, 2003; Schmitt et al., 2009) and on WPI: (Ciesla et al., 2006)). This means that the differences between unheated and heated samples were the loss of α-helix and disordered structures and the formation of intermolecular β-sheets, while the higher score on PC2 for mixtures without κ-casein can be due to a lower content in intramolecular β-sheets in the initial mixtures, as κ-casein presents limited secondary structure. PCA was also performed on heated samples alone. PC1, PC2 and PC3 explained 38, 28 and 13% of variance, respectively. Principal components did not show very strong bands and the wave numbers of these bands were hardly assigned to secondary structures (Figure 23C).

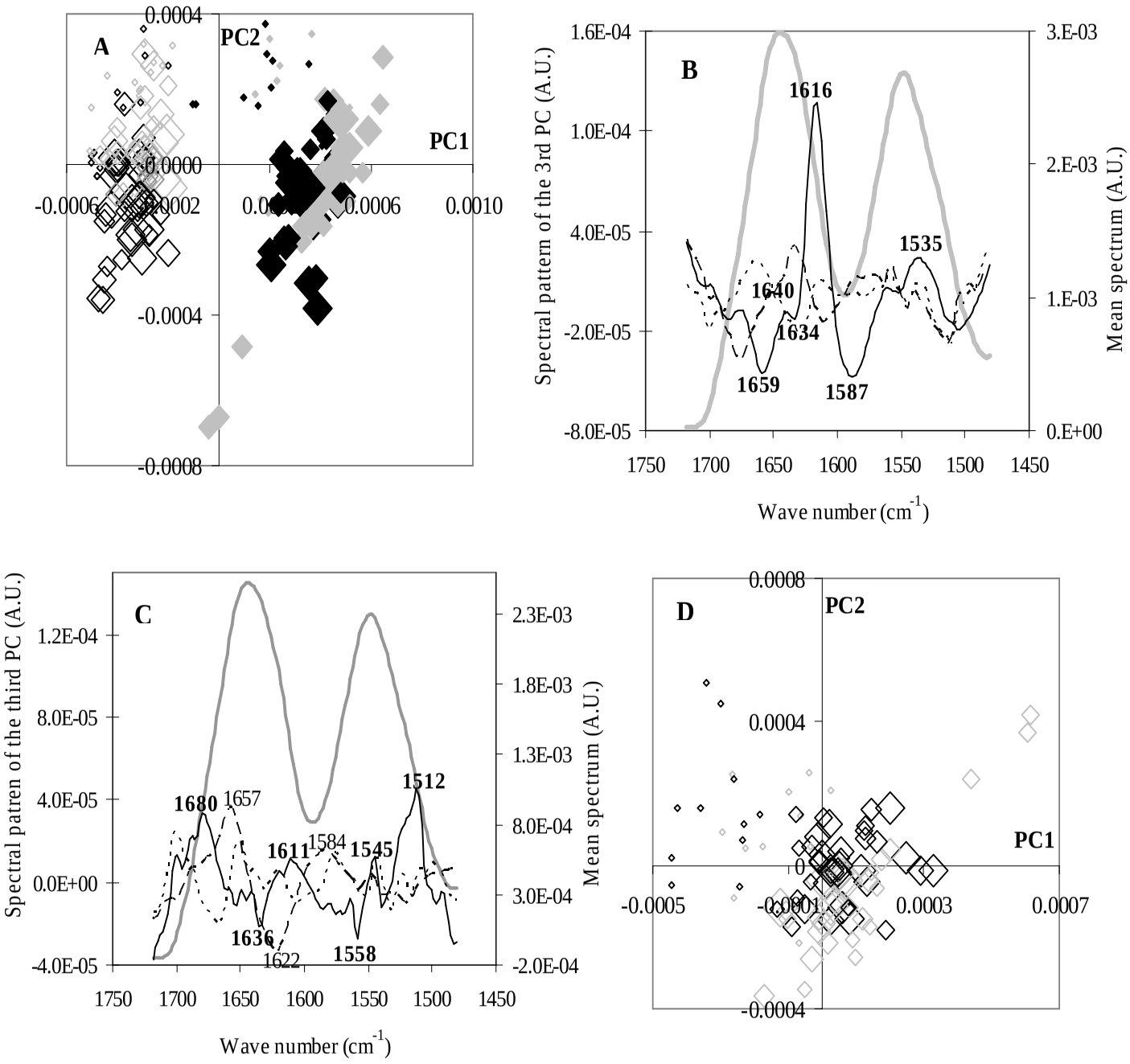

Figure 23 : Panel (A) PC1 - PC2 factorial map of the samples characterised by FTIR analysis in the region of amide I and II bands. Open and full symbols are for unheated and heated samples, respectively. Grey and black symbols are for ~ 15 and 20 g kg^{-1} WPI concentrations, respectively and the symbol size increases with the proportion in κ-casein in the original mixture. Panel (B) The mean spectrum (in grey) and the spectral pattern of PC1, PC2 and PC3 (in solid, dashed and dotted black lines, respectively) weighed by their standard deviations. Some particular wave lengths on the PC1 are indicated on the curve. Panel (C) The mean spectrum (in grey) and the spectral pattern of PC1, PC2 and PC3 (in solid, dashed and dotted black lines, respectively) weighed by their standard deviations after PCA of the heated samples. Some particular wave lengths on the PC1 are indicated on the curve (bold numbers are for PC1). Panel (D) PC1 - PC2 factorial map of the heated samples characterized by FTIR analysis. Grey and black symbols are for ~ 15 and 20 g kg^{-1} WPI concentrations, respectively and the symbol size increases with the proportion in κ-casein in the original mixture

Only the bands at 1636 cm^{-1} in PC1 and 1657 cm^{-1} in PC2 can be assigned to intramolecular β-sheets and α-helix + disordered structures, respectively. Projection of samples on PC1 and PC2 axes (Figure 23D) only shows that samples without κ-casein had negative scores on PC1 and positive ones on PC2. This means that increasing κ-casein proportion in mixtures led to a lower level in intramolecular β-sheets and α-helix + disordered structures, which can be due to the composition of initial mixtures. Although some tiny differences in complexes were found, the secondary structure of complexes in the current mixtures was very similar.

In conclusion, it appeared possible to produce a range of whey protein/κ-casein complexes with varying proportion of κ-casein were produced, without introducing major bias on other properties susceptible to affect the acid gelation of milk systems.

3.2. Role of κ-casein in the heat-induced complexes on the acid gelation of skim milk systems

The heat-induced whey protein/κ-casein complexes, having controlled proportions of κ-casein, were introduced to reconstituted whey protein-free skim milk. Figure 24 and Tableau 5 show the resulting acid-gelation behaviour of the milk systems as a function of the proportion of κ-casein in the complexes, as well as characteristics of the microstructure of the acid gels at pH 4.6. For clarity, only a reduced number of profiles are shown on Figure 24.

Tableau 5: Averages and standard deviations of typical rheological characteristics of the reconstituted 50 g kg^{-1} skim milk systems containing 40 g kg^{-1} native micellar casein (NMC) and 10 g kg^{-1} heat-induced whey protein/κ-casein complexes, acidified by 18 g kg^{-1} GDL at 35°C.

Gel characteristics	Mean ± standard deviation
tanδ max	0.43 ± 0.03
pH at tanδ max	4.96 ± 0.02

The results clearly showed that the gelation behaviour of the various reconstituted milk systems was similar whatever the proportion of κ-casein in the complexes. The pH at the onset of gelation gently decreased from 5.6 to 5.3 with increasing κ-casein ($P<0.01$), which could be

correlated to the pI of complexes (Figure 24D, P<0.01). A gelation pH value of 5.4-5.5 is usual for heated milk systems, as opposed to ~4.9 for unheated milk (Lucey et al., 1997a; Lucey et al., 1998b; Vasbinder et al., 2001). This confirmed that the presence of heat-induced complexes in the serum phase alone was enough to induce early acid gelation (Guyomarc'h et al., 2009a; Lucey et al., 1998b; Vasbinder et al., 2004).

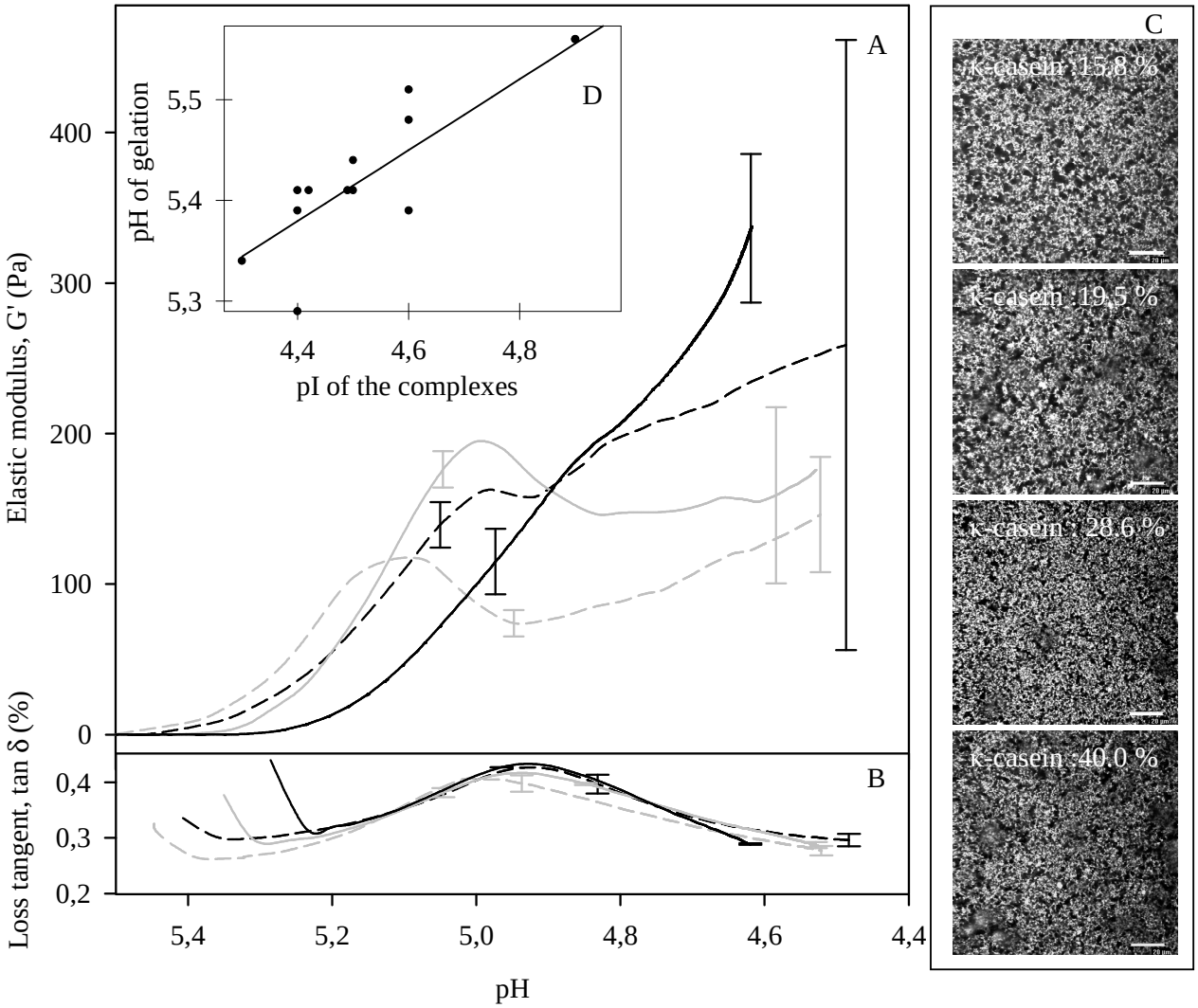

Figure 24: Development of the elastic modulus (G', panel A) and loss tangent (tanδ, panel B) of reconstituted 50 g kg^{-1} skim milk systems containing 40 g kg^{-1} native micellar casein (NMC) and 10 g kg^{-1} heat-induced whey protein/κ-casein complexes, in the course of acidification by 18 g kg^{-1} GDL at 35°C. Data are the average of 2 preparations. Selected profiles of mixtures with increasing % (w/w) of κ-casein are shown for clarity: grey dotted line = 15.8%, grey line = 19.5%; black dotted line = 28.6%; black line = 40.0%. Panel (C) shows the microstructure of the milk systems at pH 4.6 (scale bar 20 mm); insert panel (D) gives correlation of pH of gelation as a function of apparent isoelectric point (pI) of the complexes

As the pH decreased from ~6.7 to ~5.0 and passed the gel point, G' increased steadily up to ~200 Pa. As the pH further decreased towards 4.6, the acid gelation behaviour exhibited large experimental variation, with frequent shrinkage and slipping of the forming acid gel. This was unexpected and prevented complete description of the acid gel behavior. In their study, Schorsch et al. (2001) also mentioned that when pre-heated whey proteins were mixed with casein micelles, then co-heated or not, the resulting acid gels were more prone to syneresis than when native whey proteins were initially used.

On the other hand, tanδ (Figure 24B) showed a very consistent profile, marking the gel point near pH ~5.4, exhibiting a local maximum of 0.43 ± 0.03 at the average pH value of 4.96 and tending towards 0.25 as the pH reached 4.6. Again, these features are typical of heated skim milk (Lucey et al., 1997a; Lucey et al., 1998b).

Lastly, confocal micrographs of final gels (Figure 24C) did not show major differences between the structural organisation of the acid milk gels, whatever the content in κ-casein in complexes.

4. Conclusions

These results evidenced that the presence and proportion of κ-casein into the heat-induced whey protein complexes had no direct consequence on the textural and microstructural properties of the acid milk gels. In particular, the hypothesis that κ-casein might favour the interaction between the serum complexes and the casein micelles on acid gelation can probably be ruled out. Vasbinder et al. (2004) showed that this interaction probably occurred between κ-casein-free serum complexes and casein micelles, since they seemed to gel at the same moment; and the present results did not show that the presence of κ-casein helped enhance the interaction. The control of acid gelation in dairy products may therefore be obtained using whey protein substitutes to the natural whey protein/κ-casein complexes. However, further research will be needed to test whether or not the formation of whey protein/κ-casein complexes in the serum of heated skim milk helps accelerating acid gelation through depleting the casein micelle of its stabilising hairy layer of κ-casein.

Acknowledgements

Authors acknowledge the financial support from Région Bretagne, under the grant ARED 4298. The MALLS equipment was funded by the French Research Agency (ANR), European Union, Région Bretagne, Rennes Métropole and Conseil Général 35 and M. Piot for his help in cysteine determination.

IV.3. Bilan de la partie 1

Les résultats présentés dans cette partie 1 sont résumés dans la Figure 25. L'intégration de caséine-κ de 0 à 40% p/p dans les complexes thermo-induits de protéines sériques diminue le pI de ces complexes de 4.9 à 4.3. Le pH de gélification des laits reconstitués à partir de ces complexes diminue significativement de 5.6 à 5.3 avec la diminution du pI de ces complexes. De plus, l'intégration de la caséine-κ dans les complexes ne semble pas avoir d'effet sur le G' à pH 4.5 et la microstructure des gels acides formés. Pour conclure, les résultats de cette partie nous permettent de réfuter l'hypothèse d'un rôle d'ancrage de la caséine-κ présente dans les complexes et de son rôle de médiation des interactions entre les complexes et la micelle de caséines au cours de la gélification. Pour les expérimentations suivantes, la caséine-κ ne sera donc plus utilisée dans la fabrication des complexes thermo-induits modèles du lait.

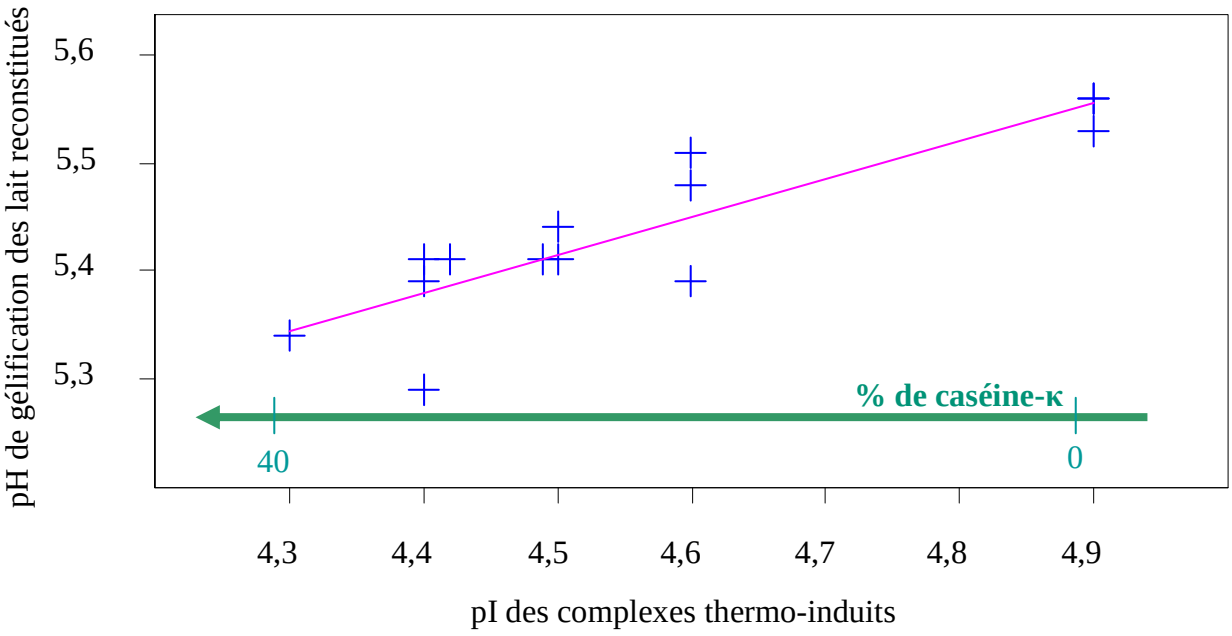

Figure 25 : pH de gélification des laits reconstitués avec des complexes thermo-induits composés de protéines sériques et de caséine-κ en proportion croissante de 0 à 40% (p/p) en fonction du point isoélectrique de ces complexes

Pour la cohérence du reste des résultats nous regrettons certains points de méthodes utilisés dans cette première partie :

➲ La détermination de l'hydrophobie avec l'ANS

Cette méthode est largement utilisée dans la littérature pour déterminer l'hydrophobie de surface des protéines. L'ANS est une molécule hydrophobe mais également chargée négativement, elle est donc aussi susceptible d'interagir avec les protéines par des intéractions électrostatiques. Il est même rapporté qu'elle est susceptible de conduire à des modifications de la structure secondaire des protéines, comme rapporté pour le cytochrome c (Ali et al., 1999). Ceci pose la question de la large utilisation de cette sonde pour la détermination des propriétés hydrophobes. L'hydrophobie de surface des complexes est restée constante pour l'ensemble des échantillons testés mais leur pI diminue avec l'ajout de caséine-κ. Les complexes riches en caséine-κ sont donc plus chargés négativement, la fixation de l'ANS sur la surface des complexes peut alors être inhibée par des répulsions électrostatiques (Alizadeh-Pasdar & Li-Chan, 2000). Selon ce raisonnement, l'hydrophobie des complexes riches en caséine-κ a pu être sous évaluée par la méthode de l'ANS. Le Prodan est une sonde non ionique qui permettrait d'éviter ce biais lié aux charges des particules mais cette sonde est encore très peu utilisée pour déterminer l'hydrophobie des protéines. Une comparaison entre l'hydrophobie mesurée par l'ANS et celle mesurée par le Prodan a été réalisée dans la deuxième partie de résultats et discussion (§V.3).

➲ La température de gélification

Les profils de formation des gels acides des laits reconstitués avec les complexes avec de la caséine-κ, montrent un décrochement (glissements, fractures) des gels dans la géométrie du rhéomètre. Ce phénomène aléatoire rend la comparaison de l'élasticité des gels impossible. La diminution de la température de gélification à 25°C permettrait de minimiser ce bais et a été envisagée dans la partie 3 (Chapitre VI). Ce choix de 35°C était pourtant dicté par un compromis entre le souci de se rapprocher des conditions de fabrication des yaourts (~ 40°C) et les limites de faisabilité, car plus la température de gélification augmente, plus la vitesse de gélification augmente, ainsi que la tendance à la synérèse des gels.

➲ Gélification des complexes seuls

De part la complexité des phénomènes intervenant au cours de l'acidification du lait, l'étude de la gélification acide d'un modèle laitier plus simple comme les suspensions de complexes en suspensions dans la phase aqueuse du lait (donc sans micelles) permettrait d'appréhender les interactions complexes-complexes s'établissant au cours de l'acidification. Le suivi de la gélification des suspensions seules a été réalisé par la suite dans les parties 2 et 3 (Chapitre V et Chapitre VI).

Chapitre V.

Résultats et discussion – Partie 2

Rôle du point isoélectrique des complexes thermo-induits sur la déstabilisation par acidification de systèmes laitiers

V.1. Introduction

D'après les résultats précédents, le point isoélectrique (pI) des complexes thermo-induits influence le pH de gélification du lait. Dans le but de mieux comprendre le rôle du pI des complexes sur les interactions protéiques au cours de la gélification acide de systèmes laitiers et selon la stratégie de la thèse, des réactions de succinylation et de méthylation sont réalisées sur les complexes thermo-induits de protéines sériques. Ces réactions permettent de modifier le pI des complexes sur une large gamme (entre 3.8 et 5.3). Nous suivrons la formation des gels des suspensions de complexes pour étudier les interactions complexes-complexes au cours de l'acidification et ceci afin de les confronter avec les interactions établies au cours de l'acidification des laits reconstitués.

Cette deuxième partie de résultats a fait l'objet d'une conférence lors du sommet mondial de la fédération internationale de laiterie (IDF) à Auckland en Nouvelle-Zélande en novembre 2010 («IDF Word Dairy Summit 2010») dont la participation a été financée par une bourse IDF France, ainsi qu'une bourse de la région Bretagne. Une conférence lors du congrès du Groupe Français de rhéologie (GFR) à également été réalisée en novembre 2010 et a fait l'objet d'un article dans le journal du GFR. Les résultats ont été également présentés sous la forme d'un poster lors du congrès Biopolymères 2010 au Croisic en France en décembre 2010. Un article a récemment été accepté pour publication dans le journal *International Dairy Journal*. L'intégralité de l'article est proposée dans ce chapitre pour présenter les résultats obtenus et leur discussion.

V.2. Article 3

Changing the isoelectric point of the heat-induced whey protein complexes affects the acid gelation of skim milk

Marion Morand[ab], Fanny Guyomarc'h[ab], David Legland[c], Marie-Hélène Famelart[ab,*]

ABSTRACT

Heat treatment of milk at 85-95°C for several minutes increases both the pH of gelation and firmness of acid milk gels, changes usually attributed to the formation of heat-induced whey protein complexes in the milk. To investigate the role of the electrostatic properties of the heat-induced complexes on the acid destabilisation of heated milk, heat-induced whey protein complexes were modified by succinylation or methylation, giving pI values ranging from 3.8 to 5.3, while size, secondary structure, surface hydrophobicity and thiol/disulfide distribution were kept reasonably constant. These complexes were added to whey-protein-free skim milk systems and the resulting acid gelation behaviour of the milk samples was evaluated. The pH of gelation of these milk samples increased significantly as the pI of the complexes increased. This demonstrated both the importance of the complexes in the acid-induced gelation of milk and the relevance of repulsive electrostatic interactions in the acid destabilisation of milk.

1. Introduction

Denaturation of whey proteins and the formation of complexes between these denatured whey proteins and κ-casein has long been recognised as a major, irreversible change that occurs on heating milk at temperatures above ~60°C (Singh, 1995). The complexes are small colloid particles assembled through hydrophobic interactions and covalent disulphide bonds, which can be found as "soluble" complexes in the serum phase of milk or as "micelle-bound" complexes attached to the surface of the casein micelles (Donato & Guyomarc'h, 2009). In yoghurt manufacture, in which heat-treatment at 85-95°C for 5-10 minutes is commonly applied, the formation of heat-induced whey protein/κ-casein complexes has been associated

with a significant increase in the pH of acid gelation, and influences the firmness and water-holding capacity of the resulting set gel at pH 4.6 (Lucey & Singh, 1998). However, despite extensive research, there is to date no detailed description of how the heat-induced whey protein/κ-casein complexes affect the colloidal interactions that influence (1) the destabilisation of the milk and (2) the mechanical properties of the final acid gel.

In unheated milk, it is known that electrostatic repulsion largely contributes to the stability of the casein micelles (Tuinier & de Kruif, 2002). Micelles precipitate when their absolute zeta potential, ξ, falls on approaching pH 4.0-4.5 (Anema & Klostermeyer, 1996; Guyomarc'h et al., 2007a). Because the whey proteins also precipitate on acidification when denatured (de Wit et al., 1988), the hypothesis soon arose that the coverage of the heated casein micelles by the micelle-bound complexes could affect their electrostatic repulsion through elevating their apparent isoelectric point (pI) from ~4.5 towards that of the major whey protein, β-lactoglobulin (pI ~5.3) (Lucey et al., 1997a; Vasbinder et al., 2001).

This proposition was supported by Alting, De Jongh, Visschers, and Simons (2002), who showed that the chemical modification of the pI of heat-induced soluble complexes of β-lactoglobulin (β-lg) or whey protein isolate quantitatively shifted their pH of acid gelation in water. However, in contrast, other studies suggested that the electrostatic properties of the heat-induced whey protein/κ-casein complexes may not be the principal origin of the heat-induced changes in the acid gelation behaviour of milk. Substituting part or all of the β-lactoglobulin with ovalbumin (pI ~4.8) (Famelart et al., 2004) or soy protein (pI ~4.0) (Roesch et al., 2004) prior to heating, could result in model skim milk systems that started to gel at pH 5.9-6.0 on acidification. Also, the measurement of the ξ potential of whey protein/κ-casein complexes isolated from the serum phase of heated skim milk showed that their apparent pI was ~4.5 (Guyomarc'h et al., 2007a; Jean et al., 2006), which no longer linked the pH of gelation of heated milk directly to the pI of the native β-lactoglobulin. Donato & Guyomarc'h (2009) further reminded that the heat-induced whey protein/κ-casein complexes also exhibit significant surface hydrophobicity and suggested that the increased hydrophobic attraction opposed electrostatic repulsion on a larger pH range, thus inducing destabilisation of the milk at higher pH values on acidification.

Despite these propositions, this overview therefore shows that the actual role of the electrostatic properties of the heat-induced whey protein/κ-casein complexes on the acid gelation of milk has not yet been thoroughly evaluated. Recently, Morand, Guyomarc'h, Pezennec, and Famelart (2011b) decreased the apparent pI of heat-induced soluble complexes from 4.6 to 4.3 through increasing their proportion of κ-casein from 0 to 40% (w/w) and showed that this decrease in pI correspondingly decreases the pH of acid gelation of a heated skim milk system. However, the range of pI modifications was too restricted to draw any general conclusion.

In their review, Donato & Guyomarc'h (2009) proposed that the heat-induced whey protein/κ-casein complexes altered the stability of the casein micelles and hence their acid gelation behaviour, through interacting with their surface on heat-treatment (micelle-bound complexes) or early during acidification (soluble complexes), thus modifying the colloidal interactions. In line with this hypothesis, Morand et al. (2011a) further proposed that the role of the heat-induced whey protein/κ-casein complexes in affecting the interactions in acidified skim milk could be investigated using model soluble complexes with modified interaction properties as a means to modify the colloidal interactions between the casein micelles.

Following this strategy, the present paper reports on the effect of modifying the isoelectric point of model heat-induced soluble whey protein complexes on the acid gelation behaviour of skim milk. Since experimental evidence has shown that κ-casein in the heat-induced complexes did not play a particular role in the acid gelation of milk (Morand et al., 2011b), the chosen model complexes were composed only of denatured whey proteins. The pI of the complexes was either decreased using the conversion of their amino groups into carboxyl groups (through succinylation) or increased using the neutralisation of their carboxyl groups (through methylation). The modified complexes were characterised then combined with whey protein-free skim milk to compose a model heated skim milk, the acid gelation behaviour of which was monitored using rheometry and confocal laser scanning microscopy.

2. Materials and Methods

2.1. Materials

Native micellar casein (NMC, 870 g kg^{-1} of proteins on a dry basis, with whey protein level being 5% of the total protein) was prepared as described by Schuck et al. (1994). Briefly, raw milk was skimmed at ~50°C then microfiltered through a 0.1-µm cut-off ceramic membrane, diafiltered, concentrated by evaporation and spray-dried. The microfiltration permeate, containing the native whey proteins, was concentrated by ultrafiltration at ~ 50°C using an 8-kDa cut-off ceramic membrane, diafiltered and freeze-dried to yield a native whey protein isolate (WPI, 970 g kg^{-1} of proteins on a dry basis). The protein composition of the WPI as given by reverse phase-high performance liquid chromatography (RP-HPLC) was, in % of the total area (method by Jean et al., 2006): ~80% whey proteins, ~10% β-casein and minor amounts of κ-, α_{s1}- and α_{s2}-caseins. The milk ultrafiltration permeate (MUF) resulting from UF-concentration of the whey protein fraction was collected and stored at 5°C after addition of 0.2 g L^{-1} sodium azide.

Pure bovine β-lactoglobulin (β-lg, 954 g kg^{-1} of proteins on dry basis, with β-lg level as 97% of the total protein) was purchased from a commercial supplier (confidential prototype). All other reagents were of analytical grade.

2.2. Preparation of the heat-induced complexes

A control whey protein complex (WPIA 0) was prepared as described by Vasbinder van de Velde, and de kruif (2004). Briefly, a 90 g kg^{-1} solution of WPI in deionised water with 0.2 g kg^{-1} NaN_3 was adjusted to pH 7.5 then heated at 68.5°C for 2 h. The heat-induced complexes thus produced were essentially composed of whey proteins, as evidenced by RP-HPLC analysis of complexes isolated by size exclusion chromatography (not shown). The heat-induced complexes were then diluted to ~45 g kg^{-1} with deionised water and subsequently modified using succinylation or methylation as described by Alting et al. (2002) or Broersen Weijers, de Groot, Hamer, and De Jong (2007). Succinylation was performed at pH 8 with succinic anhydride at concentrations ranging from 5 to 200 mM for 2 h (WPIA-1, -2, -3,

Tableau 6). Methylation was performed at pH 6 for 2 h in presence of 1M methylamine and N-3-dimethylaminopropyl-N-ethylcarbodiimide hydrochloride (EDC) at concentrations of 50-300 mM (WPIA+1, +2, Tableau 6). For mass spectroscopy analysis, ~45 g/kg solutions of unheated βlg or WPI were also succinylated or methylated following the same protocol. After the reaction period, the solutions were extensively dialysed (6-8 kDa, Medicell International Ltd., London, UK) against deionised water adjusted to pH 7.5 with 0.2 g kg^{-1} NaN_3 added as preservative, to eliminate the residual reactants. The final total protein content of the complexes solutions was determined using absorbance at 280 nm and an extinction coefficient value of 1.046 L g^{-1} cm^{-1} for the WPIA (Morand et al., 2011b).

2.3. Evaluation of the degree of modification

2.3.1. Chromogenic OPA assay to determine the degree of succinylation

This assay is based on the reaction between ortho-phthaldialehyde (OPA) and the free primary amino groups of proteins, which, in the presence of 2-dimethylaminoethanothiol hydrochloride (DMA), yields alkyl-iso-indole derivates that absorb light at 340 nm (Church et al., 1983). A fresh OPA solution was prepared by weighting 40 mg of OPA into 1 mL of ethanol, with addition of 0.5 g of anhydrous Borax, 200 mg of DMA, 0.7 g of sodium dodecyl sulphate, and deionised water to a total volume of 50 mL. Absorbance at 340 nm was measured on 3 mL of OPA solution to which 35 µL of WPIA solution were added. A calibration curve was obtained by adding 10, 20, 30, 40, 80 or 100 µL of 2 mM L-leucine to 3 mL of the OPA solution. Each determination was performed twice.

2.3.2 Liquid Chromatography coupled with Mass Spectroscopy (LC/MS)

In order to help identification of the mass/charge peaks using a simplified mass spectrum signature, solutions of β-lg in deionised water were succinylated or methylated as described previously and analysed with LC/MS as well as the modified and control WPI samples. Unheated β-lg and WPI were first diluted at 9 g L^{-1} in a trifluoroacetic 0.01% (v/v) - methanoic acid 0.8% (v/v) solution. Reverse Phase-High Performance Liquid Chromatography separation was performed on an Agilent HP 1100 system equipped with a Luna C18(2) column

(Phenomenex, Le Pecq, France) of 2 mm inner diameter, 10 cm length and 3 µm bead size. Buffer A was 0.106% (v/v) trifluoroacetic acid in water and buffer B was 80% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid in water. The analysis was performed at 40°C with a gradient of buffer B increasing from 35 to 80 % (v/v) in 15 min. The flow-rate was 0.250 mL min^{-1}, 12 µL of sample (~100 µg of protein) was injected and detection was at 214 nm. LC/MS analysis was performed using a mass spectrometer API III+ (Sciex, Canada) that comprises a triple quadripole equipped with an atmospheric pressure ionisation source operated at 4800 V. Orifice voltage (OR) was 80 V. The nebulizer pressure was ~0.5 MPa and the curtain gas flow was 1.2 L min^{-1}. The theoretical molecular masses, M_w, of β-lg A and B are 18 364 and 18 278 Da, respectively, while those of the succinylated proteins were M_w+N*100 Da and those of the methylated proteins were M_w+N*14 Da, with N the number of attached moieties.

2.4. Physico-chemical properties of the complexes

As we aim at studying only the effect of the isoelectric point of the complexes, we had to check that chemical graftings did not modify other properties of complexes. Therefore, the physico-chemical properties of the WPIA complexes, namely their apparent isoelectric point, size, secondary structure, and their contents of surface and total free thiol groups and of total cysteine residues were measured using the same methods as described by Morand et al. (2011b).

The apparent isoelectric point (pI) of the heat-induced whey protein complexes was determined using interpolation to 0 of the experimental measurement of the complexes' electrophoretic mobility (in µm cm V^{-1} s^{-1}), while the pH of the MUF was varied from ~2 to 6.7 and corrected for calcium ions (Morand et al., 2011b;(Mekmene et al., 2009). The electrophoretic mobility was measured at 50 V on a Zetasizer nanoZS equipment (λ=633 nm, Malvern Instruments, Orsay, France) as in Morand et al. (2011b).

Briefly, the hydrodynamic diameter D_h of the complexes was determined at 20°C in MUF using dynamic light scattering at a fixed angle of 173° on the same Zetasizer nanoZS Malvern as for electrophoretic mobility measurement. The data were converted into a particle size distribution using the non-negative least-squares (NNLS) modelling routine and the refractive

index of water (1.330). A major peak was found, of which the mean (D_h) and the width were calculated using the Stokes-Einstein relationship (viscosity of water = 1.0031 mPa s).

The protein surface hydrophobicity index was determined using a non-ionic probe, the 6-propionyl-2-(N,N-dimethylamino)-naphthalene (PRODAN, Molecular Probes, Life Technologies SAS**,** Villebon sur Yvette, France). The method described by Alizadeh-Pasdar & Li-Chan (2000) was adapted. Briefly, a stock solution of 0.7 x 10^{-3} M PRODAN was prepared in methanol. Samples were diluted in a phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 0.02% NaN_3, pH 7) to obtain 4 mL of solution at 0.02-0.1 g of protein kg^{-1} (5 concentrations). Ten µL of the stock solution of PRODAN were added. The fluorescence was measured (excitation λ=365 nm, emission λ=465 nm, slits 5 nm) with a LS50B spectrophotometer (Perkin Elmer, Saint Quentin-en-Yvelines, France) after 15 min in the dark. An index of the protein surface hydrophobicity, PSH, was defined as the initial slope of a plot of the fluorescence versus protein concentration.

The levels of accessible thiol groups (surface SH), total free thiol groups (SH) and total cysteine residues (S) of the WPIA complexes were assayed using the 5,5'-dithio-bis-2-nitrobenzoïc acid (DTNB) reagent method (Ellman, 1959) on aliquots diluted in non-dissociating Tris buffer at pH 7.4, in dissociating urea-citrate-sodium dodecyl sulphate-Tris buffer at pH 7.4 or in dissociating buffer in the presence of β-mercaptoethanol (Sigma-Aldrich, St Quentin Fallavier, France) (Morand et al., 2011b), respectively.

The secondary structure of proteins was assessed by Fourier Transform Infra-Red measurements (FTIR) in the Attenuated Total Reflectance (ATR) mode, as described by Morand et al. (2011b). A principal component analysis (PCA) was performed on the spectra of the WPIA, using the R package (R 2.9.2. Foundation for Statistical Computing). FTIR coupled with PCA is a high-sensitivity global method to control, in a complex environment, that complexes were the same (Carbonaro & Nucara, 2010).

2.5. Acid gelation behaviour of the WPIA

First, the whey protein complexes were extensively dialysed (6-8 kDa, Medicell International Ltd., London, UK) at 4°C against commercial UHT half-fat milk in order to replace their solvent phase with milk permeate. The resulting protein concentration was determined spectrophotometrically, then each control or modified WPIA suspensions was standardized to 20 g kg^{-1} total protein using MUF. The standardized suspensions were equilibrated at 35°C prior to acid gelation by addition of 17 g kg^{-1} glucono-δ-lactone (GDL). Formation of the acid gels was monitored at 35°C, using an AR2000 rheometer (TA Instruments, Guyancourt, France) equipped with a cone-plane geometry and using the oscillatory mode at a frequency of 1 Hz and a strain of 0.1%. The pH was recorded with time using an Inlab 415 electrode (Mettler-Toledo, Viroflay, France) placed in a sample aliquot incubated in a water bath at 35°C. Gelation time was defined as the point at which G' > 1 Pa.

2.6. Acid gelation behaviour of reconstituted heated milk systems

2.6.1. Reconstitution of the milk systems

A stock suspension of NMC was reconstituted at 65 g kg^{-1} in MUF with 0.2 g kg^{-1} NaN_3 at 40°C and stored overnight at 4°C. The NMC suspension, milk-dialysed WPIA complexes and MUF were mixed as to reach a total protein content of 50 g kg^{-1} and a NMC to complexes ratio of 80:20 (w/w). The resulting systems were regarded as reconstituted models of heated skim milk with modified or control whey protein heat-induced soluble complexes.

The reconstituted heated milk systems were equilibrated at 35°C and labelled using 0.2 g kg^{-1} rhodamine B isothiocyanate solution (4 g L^{-1} RITC in dimethylsulfoxyde, Sigma-Aldrich, St Quentin Fallavier, France) prior to acid gelation.

2.6.2. Rheological measurement of the formation of acid milk gels

The reconstituted milk systems at 35°C were acidified using addition of 18 g kg^{-1} GDL. Formation of the acid milk gels was monitored at 35°C using the same equipments and methods than for the WPIA. Each reconstituted milk system was prepared twice, and acid gelation was monitored on at least two aliquots of each reconstituted system. The rheological

properties were then characterised at large deformations. Final gels (pH 4.5) were subjected to a dynamic oscillation step at log-increasing stress amplitudes ranging from 0 to 3.5 kPa. The yield stress (in Pa) was defined as the point when the G' started to decrease. The yield stress was taken at the intersection between the linear regressions of the viscoelastic domain and of the breakdown domain.

2.6.3. Compression test of the final acid milk gels

Immediately after GDL dispersion, 5 x 10-mL aliquots of each suspension were aliquoted and incubated at 35°C until pH 4.5, then immediately cooled to 4°C and kept overnight at that temperature. Compression tests were performed at 4°C with the 4501 INSTRON universal testing machine using the series IX software (Instron SA, Guyancourt, France) fitted with a 10 N sensor and a 11-mm diameter, 45-mm height plastic cylinder geometry. The procedure involved a penetration step of 10 mm into the sample at a speed of 120 mm s^{-1}. The initial slope of the force versus depth of the cylinder in the gel (in N mm^{-1}) was used as the apparent stiffness of the gels.

2.6.4. Image analysis of the acid milk gels microstructure

Immediately after GDL dispersion, one drop of the RITC-labelled milk system was deposited on a concave slide, covered by a cover slip sealed with nail varnish and incubated at 35°C. When the pH reached 4.5, the slide was introduced to a TE2000-E inverted confocal laser scanning microscope (CLSM) equipped with a Nikon C1i imaging system (Nikon, Champigny-sur-Marne, France) with an He-Ne laser emitting at 543 nm. A Nikon 100x oil-immersion objective with a 1.40 numerical aperture was used. The emitted light was recorded at 590 ± 25 nm. The software used for image acquisition was EZ-C1 version 3.40 (Nikon). Digital images were recorded at 15 µm depth into the sample. Dwell time was 10.56 µs per pixel. Pinhole diameter and all other settings were also kept constant. Image brightness was auto-adjusted using the software EZ-C1 FreeViewer, version 3.20 (Nikon). Each image was digitized as a 512 x 512 pixels matrix with grey levels coded between 0 (black) and 255 (white). The resolution was 0.249 µm per pixel in each direction, resulting in a field of view equal to 127.3 x 127.3 µm.

Histogram equalization was applied on all images to remove illumination variations. Grey-level granulometric methods from mathematical morphology were applied as described by Devaux et al. (2008). The method consists of applying image filters (defined by a structuring element of given shape and size) that make structures smaller than the structuring element disappear. By applying filters with increasing sizes of structuring element, and by considering the derivative of the sum of grey levels obtained at each sizes, one obtains a distribution of the sizes of grey regions in the image. Morphological closing was used, making dark regions disappear. The structuring element was a square, with side-lengths ranging from 0.75 to 50 microns. Principal component analysis (PCA) was performed on these curves for all images, using the R package (R 2.9.2. Foundation for Statistical Computing).

3. Results and discussion

3.1. Chemical modification and change in the pI of the heat-induced complexes

The success of each chemical modification reaction was evaluated for unheated β-lg and WPI using mass spectrometry. B-lg, as the major whey protein in WPI, was used as a spectrum marker in the intricate mass spectrum signatures of the WPI samples. In agreement with expectations, the M_w of the β-lg variants were found to be about 18 368 and 18 281 Da in all samples, allowing for a standard error of ±5 Da. The mass spectroscopy analysis evidenced the increase in the M_w of the genetic variant A and B in increments of 100 or 14 Da after the succinylation or methylation, respectively, of β-lg in pure form and in WPI (Tableau 6). These results were in agreement with the addition of units of succinic acid (M_w 100 Da) or methylamine (M_w 14 Da) on the protein. In the case of succinylation, the mass spectra showed a range of 7-13 succinic moieties attached to each variant in β-lg and WPI, while the methylated samples were shown to have 0 to 28 added methyl groups (Tableau 6). These results confirmed that the succinylation and methylation reactions were successfully applied to the native proteins.

Tableau 6 : Characterisation of protein modification for ~45 g kg^{-1} unheated (Blg or WPI) or heated (WPIA) β-lactoglobulin and whey protein isolate using either succinylation (-) or methylation (+) at different levels (0: non-modified, 1: low modification, 2: medium modification and 3: extreme modification).

sample	Succinic acid (mM)	EDC[a] (mM)	Molecular mass of β-lg or number of grafted groups[b]		Degree of modification by OPA[c] assay (%)	Apparent pI[d] (pH units)	pH of acid gelation of WPIA (pH units)	pH of acid gela of heated skim (pH units)
			Variant A	Variant B				
Blg0	0	0	18 368 Da	18 281 Da	0	nd	nd	nd
Blg-3	100	0	7 to 13	7 to 11	94 n=1	nd	nd	nd
Blg+2	0	300	0 to 25	0 to 21	nd	nd	nd	nd
WPI 0	0	0	18 368 Da	18 281 Da	0	nd	nd	4.8 ±0.0 n=2
WPI-3	100	0	7 to 13	7 to 14	95 n=1	nd	nd	nd
WPI+2	0	300	0 to 28	0 to 19	nd	nd	nd	nd
WPIA 0	0	0	nd	nd	0	4.9 ±0.0 n=5	5.6 ±0.1 n=2	5.5 ±0.0 n=5
WPIA-1	5	0	nd	nd	32 n=1	4.3 ±0.1 n=4	5.1 ±0.0 n=3	5.4 ±0.0 n=3
WPIA-2	10	0	nd	nd	64 ±41 n=2	3.8 ±0.2 n=5	4.8 ±0.1 n=3	5.2 ±0.2 n=3
WPIA-3	40-200	0	nd	nd	94 ±7 n=4	3.2 ±0.0 n=7	nd	nd
WPIA+1	0	50-200	nd	nd	nd	5.1 ±0.1 n=5	5.8 ±0.1 n=2	5.8 ±0.1 n=2
WPIA+2	0	300	nd	nd	nd	5.3 ±0.2 n=3	5.8 ±0.1 n=2	5.6 ±0.1 n=2

n: number of samples
nd: not determined.
[a] EDC: N-3-dimethylaminopropyl-N-ethylcarbodiimide hydrochloride.
[b] range of molecular masses detected or unambiguously detected adducts in case of poor signal (+100 Da for succinate and +14 Da for methylamine); A and B are the major genetic variants of β-lactoglobulin identified by LC/MS. n, number of statistical samples.
[C] OPA: ortho-phthaldialehyde.
[d] pI : apparent isoelectric point.

The degree of modification of the succinylated complexes was furthermore assayed by the OPA method. OPA assay of the residual ε-amino groups in the control and modified complexes showed that 32-94% of them were converted into carboxyl groups by the succinylation reaction (Tableau 6). These levels were in agreements with mass spectrometry results for native proteins. The methylation of native proteins was checked by mass spectroscopy, but methylation of complexes was only observed through the modification of their pI values (see the following section), due to the difficulty of implementing the chromatographic separation step of the assay by Koster & de Jongh (2003) to heat-induced complexes. The reactions were thereby presumed to be successfully applied to the complexes.

After equilibration of the modified and control complexes in MUF at 20°C, their apparent isoelectric point, pI, was determined. The pI value of the control sample (WPIA 0) was found to be ~4.9 (Figure 26, Tableau 6; see also Morand et al. (2011b). Tableau 6 and Figure 26 show that increasing the amount of succinic anhydride from 0 to 200 mM in the 45 g kg^{-1} WPIA suspensions induced a decrease in the apparent pI of the complexes from ~4.9 to ~3.2. The maximum shift in pI obtained for the succinylated complexes was therefore -1.7 pH units and was shown to level off on addition of over 40 mM succinic anhydride (WPIA-3: 94% modification, Tableau 6). The absolute value was only slightly larger than that calculated by Alting et al. (2002) for the addition of 70 mM succinic anhydride to a solution of heat-induced complexes of β-lg (96% modification, pI-shift -1.3 pH units). Interestingly, the pI of the low succinylated complex (WPIA-1) was near to that of the natural whey protein/κ-casein complexes of heated skim milk (pI = 4.5, Jean et al., 2006). In contrast, the pI of the complexes increased from ~4.9 to ~5.3 with increasing of EDC addition (Figure 26, Tableau 6), yielding a maximum pI-shift of +0.4. The pI increased most on addition of 50 mM EDC (pI ~5.1), then levelled off at larger EDC addition up to 300 mM. Although these results confirmed that the methylation reaction succeeded in increasing the pI of the complexes, the amplitude of the pI-shift was lower than that obtained with succinylation. This was also observed by Rowley et al. (1979) or Simons et al. (2002) when they methylated or succinylated native β-lg. This is partially explained because methylation only neutralises carboxyl groups on the protein complex, while succinylation substitutes one acidic group for one amine. Furthermore, as above mentioned, the level of modified protein was very low, no matter the amount of EDC

added. This means that a proportion of the complexes may have retained their initial pI of ~4.9 in the treated samples or that only a small amount of carboxyl groups per complex were neutralized, which may have affected the macroscopic determination of their apparent pI using light-scattering.

At pH 6.7, clear differences between the surface charge of the control and modified complexes could also be observed (Figure 26). The succinylated samples were more negatively charged, with a maximum electrophoretic mobility of about -1.54 µm cm V^{-1} s^{-1}, than the control WPIA 0 (electrophoretic mobility of -0.92 µm cm V^{-1} s^{-1}) and the methylated samples (electrophoretic mobility of -0.87 µm cm V^{-1} s^{-1}). These observations further confirmed that negative charges have been added on the complexes by succinylation and that negative charges have been removed by methylation.

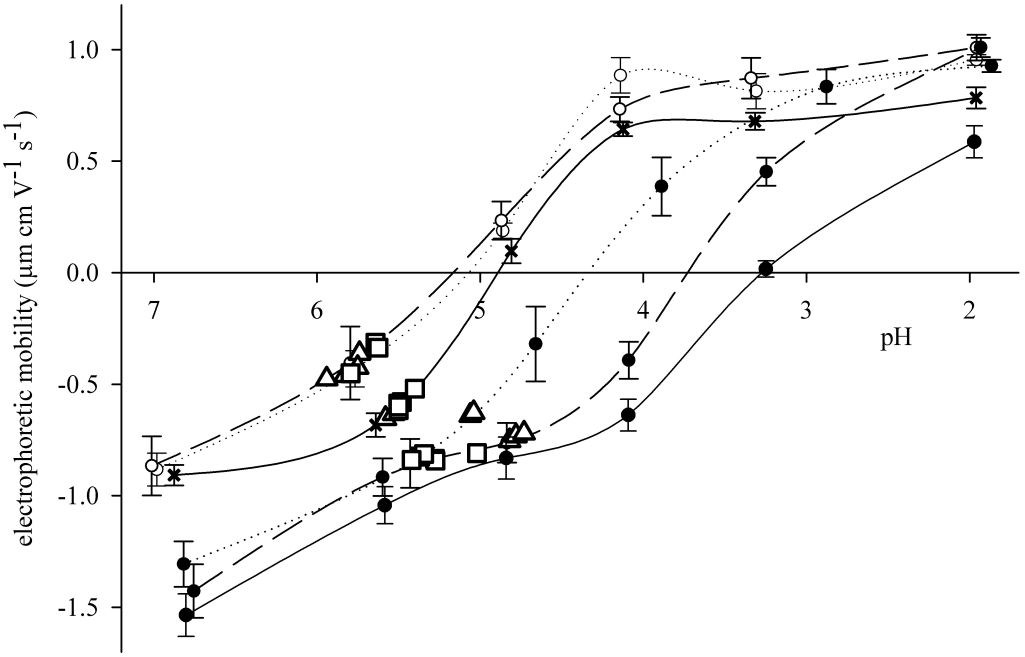

Figure 26 : Electrophoretic mobility of heat-induced whey protein complexes measured at different pH in acidified Ca-MUF by zetametry. The cross represents the control complexes, the full circles the succinylated ones (WPIA-) and open circles the methylated ones (WPIA+). Dotted, dashed and solid lines are respectively for low (WPIA-1 or +1), intermediate (-2 or +2) and extremely modified complexes (-3). Symbols indicate the values of the electrophoretic mobility of the complexes at the pH of gelation of the suspensions of complexes (△) and at the pH of gelation of the milk systems (□)

3.2. Physico-chemical properties of the modified heat-induced complexes

In agreement with the findings of Alting et al. (2002), the hydrodynamic diameter D_h of the complexes increased with the intensity of modification. While the control WPIA 0 exhibited an average D_h value of 86 ± 5 nm, low modification levels (e.g., WPIA-1, -2 and +1) induced an increase in the D_h of the complexes to ~115 ± 30 nm ($P<0.05$) and extreme modification levels (e.g. WPIA-3 and +2) could increased this value up to ~240 ± 80 nm ($P<0.01$, Figure 27). These values were somewhat larger than the maximum D_h of 176 nm reported by Alting et al. (2002) for 93% succinylated WPIA complexes. On succinylation of ~70% of the total ε-amino groups in raw skim milk, Lieske (1999) also observed an increase in the D_h of the casein micelles of ~15% at pH 6.7. In their paper, Alting et al. (2002) suggested that the increase in the D_h of the succinylated complexes was caused by the increase in electrostatic repulsion within the particles, which would loosen their structure and increase their voluminosity.

Previous reports have suggested that larger complexes yielded firmer acid gels (Guyomarc'h et al., 2003a; Puvanenthiran et al., 2002). The size of the heat-induced complexes is also quite likely to play a role in the final microstructure of the acid gel, e.g., by affecting pore size and connectivity (Kalab et al., 1983; Parnell-Clunies et al., 1987). Since these changes are therefore likely to introduce a bias in our study, it was decided to only use complexes of maximum diameter 130 nm, i.e., having modified pI in the range 3.8-5.4 (Figure 27, hatched zone).

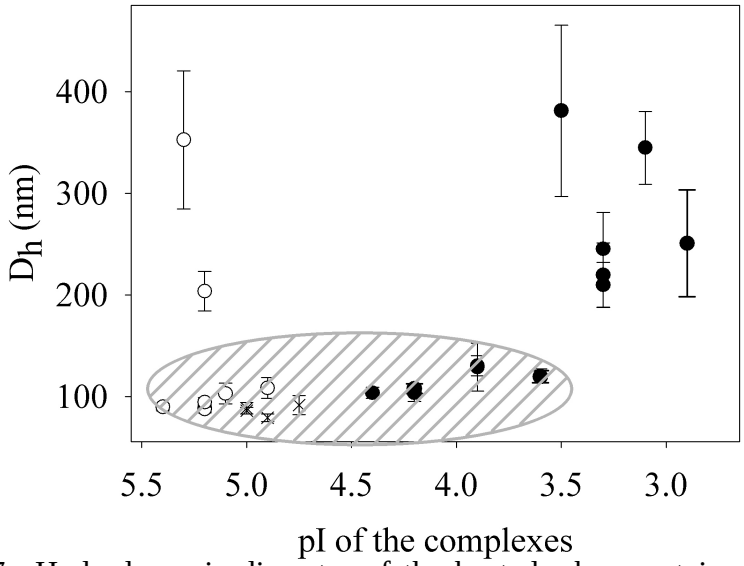

Figure 27 : Hydrodynamic diameter of the heated whey protein mixtures (D_h in nm) as a function of the pI of complexes. The crosses represent the control complexes, the full circles are for succinylated complexes and the open circles for methylated complexes. The hatched area represents the samples with D_h inferior to 130 nm, selected for acid gelation.

The surface hydrophobicity index (PSH) of the complexes was determined using the PRODAN probe. The calculated PSH values did not show any significant difference between samples whatever their pI ($P>0.05$, not shown). Broersen et al. (2007) also did not report significant changes in the surface hydrophobicity of either on succinylated or methylated ovalbumin using PRODAN.

The total cysteine content of all samples was about 296 ± 40 µmol g^{-1} total protein and, as expected, did not show significant differences between samples and control whatever the modification ($P>0.05$). The total free thiol content, SH, was about 13, 8 and 3 ± 2 µmol g^{-1} total protein for the control, the most succinylated and the most methylated complexes, respectively. It is possible that succinylation occurred to some extent on sulfhydryl rather than amino groups (Aitken & Learmonth, 2002b). On the other hand, SH groups also represented less than 5% of the total cysteine content in all the samples. This result indicates that the majority of the cysteines were involved in disulfide bridges. In conclusion, regarding the large amount of disulfide bridges, the thiol composition of the modified complexes as evaluated using the Ellman method was not considered different to that of the control, WPIA 0.

Secondary structures of the control and modified complexes were studied by FTIR and PCA. PC1, PC2 and PC3 accounted for 40.6, 34.4 and 13.6% of the variance, respectively. Figure 28A shows that the spectral pattern of PC1 presented a small positive peak at 1562 cm^{-1} in the amide II band (1480-1575 cm^{-1}) and a small negative peak at 1633 cm^{-1} that are assigned to CN stretching or NH bending and to intramolecular β-sheets, respectively (Carbonaro & Nucara, 2010) in association with heat-induced denaturation of the β-lg (Boye et al., 1996). The score on PC1 was well correlated with the pI of the complexes ($P<0.01$, Figure 28B) with a higher positive peak at 1562 cm^{-1} and a higher negative peak at 1633 cm^{-1} for the succinylated complexes. Since Boye et al. (1996) also found a loss of the 1635 cm^{-1} band as the pH of heating was increased (i.e., when the β-lg was most electronegatively charged), one could suspect that increased negative repulsions may have broken up some intramolecular β-sheets in the succinylated complexes. However, the maximum spectrum variation obtained at 1633 cm^{-1} represented only about 4% of the total intensity of the mean spectrum at 1633 cm^{-1}. No major peak could be identified on PC2 or PC3 (not shown).

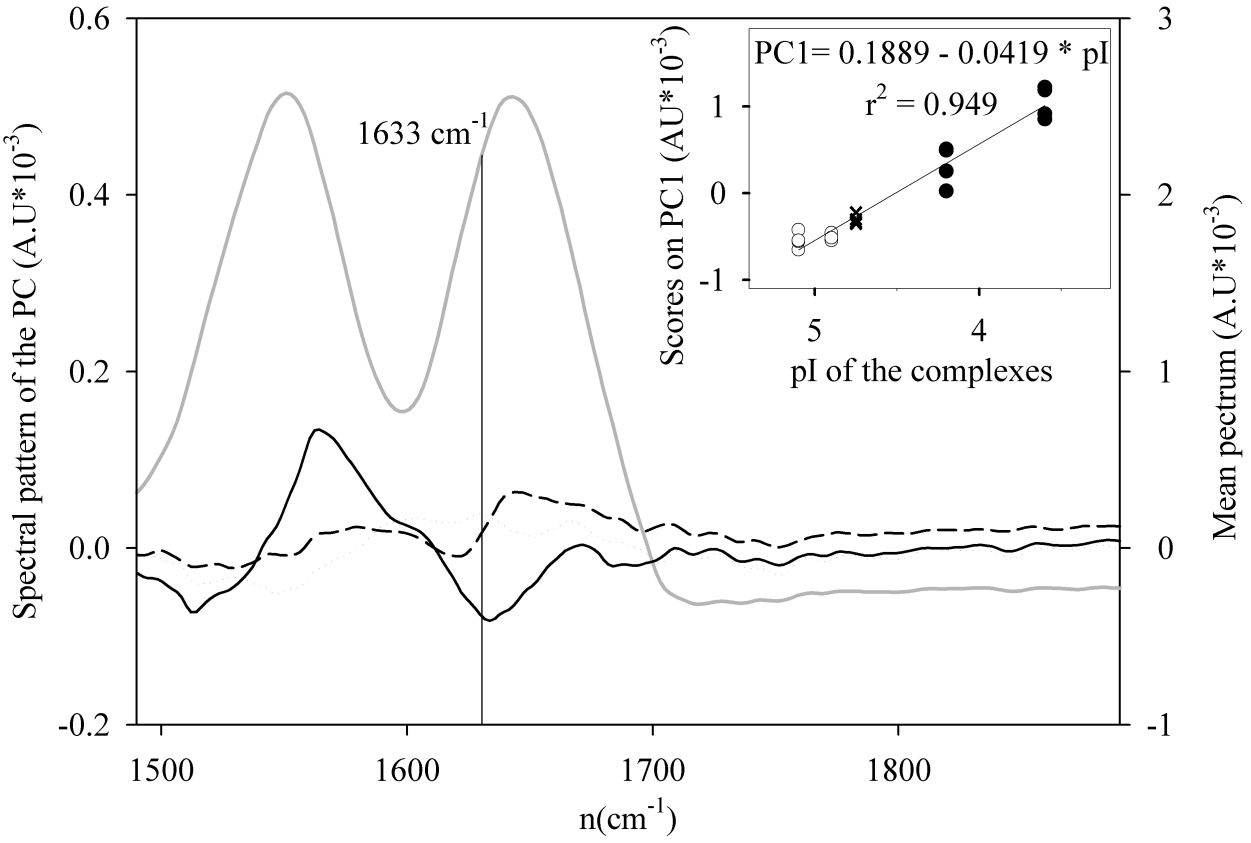

Figure 28 : (A) The mean spectrum (in grey) and the spectral pattern of PC1and (in solid and dashed black lines, respectively) weighted by their standard deviations of the samples characterized by FTIR analysis in the amide I and II bands. The scales of mean spectrum and PC are on right and left axis, respectively; (B) Scores on PC1 of the samples as a function of pI. The crosses represent the control complexes, the full circles are for succinylated complexes and the open circles for methylated complexes. The inserted figure shows the linear regression equation and the squared linear correlation coefficient (r^2)

In conclusion, the surface charge and the pI of the complexes were significantly modified by succinylation or methylation. The other properties, namely the size, the surface hydrophobicity, the thiol composition and the secondary structure were marginally affected by these modifications. It therefore seemed possible to produce a range of heat-induced whey protein complexes with varying pI from 3.8 to 5.4, without introducing any major bias that could otherwise affect their acid gelation behaviour.

3.3. Effect of heat-induced complex modifications on acid gelation of milk

3.3.1. Acid gelation of suspensions of complexes alone

The influence of electrostatic interactions in the course of acid gelation was first evaluated on the complexes alone. Figure 29 shows that the pH of gelation of 20 g kg-1 suspensions in MUF was well correlated with the apparent pI of complexes ($P<0.01$). As a result, reducing the pI of the complexes from 5.4 to 3.8 induced a delay in the pH of onset of acid gelation from 5.8 to 4.8 (Tableau 6 and Figure 29). However, the gelation kinetics for these systems did not obey similar relationships. Instead, it seemed that any modification (either succinylation or methylation) accelerated the increase in G' on acidification (Figure 29A). At pH 4.5, whatever the type and level of chemical modification, the G' of the modified complex gels (between 100 and 300 Pa) was higher than that of the control gel (100 Pa). Only the final G' of WPIA-2 (50 Pa) was lower than the control, but as it only gelled at pH 4.8, gel formation was not completed at pH 4.5. At pH 4.5, the tan δ of the control gels was lower than that of the succinylated or methylated ones ($P<0.01$). For the same reason as previously, the final tan δ of WPIA-2 was higher than 0.2. This result indicated that the chemical modifications induced firmer but somewhat more viscous-like gels. The grafted groups may have induced steric obstruction that disturbed the organisation of the gel. Also, the indeed modifications may have affected the organisation of the surrounding hydrogen bonds and consequently the cohesiveness of the protein structures and/or the binding of water to the protein. In conclusion, modifications of the surface charge of the complexes induced corresponding shifts of the pH of gelation of the acidified suspensions. The modifications furthermore seemed to accelerate the formation of the gel and to yield firmer but more flowable gels.

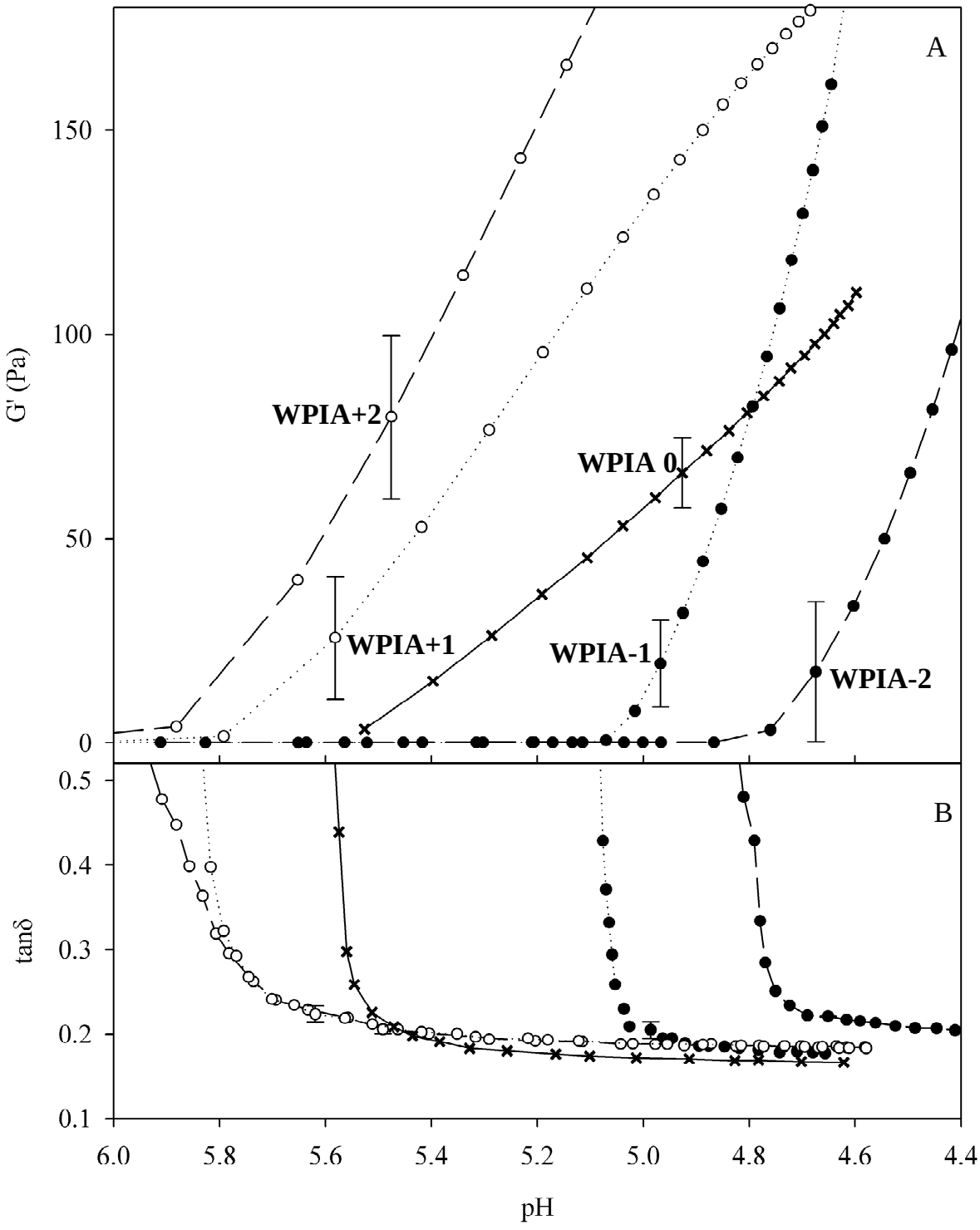

Figure 29 : Development of the elastic modulus (G' in Pa) (A) and loss tangent (tan δ) (B) of heated whey protein mixtures adjusted at 20 g kg^{-1} in MUF, in the course of acidification by 17 g kg^{-1} GDL at 35°C

3.3.2. Acid gelation of recombined model milk systems

The modified and control heat-induced whey protein complexes were introduced into reconstituted whey protein-free skim milk to yield model heated milk systems, Figure 30 shows the resulting acid gelation behaviour of milk systems as a function of the pI value of the complexes. The results clearly showed that reducing the pI of the complexes from 5.4 to 3.8 induced a delay of the onset of the acid gelation from 5.8 to 5.2 when introduced to the milk systems (Figure 30, Tableau 6, $P < 0.01$). This result underlined the relevance of the heat-induced whey protein complexes as driving elements for initiation of the building of the acid milk gel. The modification of the pI of the complexes, however, generated lower shifts in the pH of gelation of the milk systems than of the suspensions of complexes ($\Delta pH_{gelation}$ of 0.6 and 1.0 pH unit, respectively, for a ΔpI of 1.5 pH unit, Figure 30 and Tableau 6).

Milk with WPIA-1 complexes exhibited the highest final G' value, reaching about 1000 Pa at pH 4.5 (Figure 30A). Although the acid gels with methylated complexes had more time for rearrangement on acidification (since they gelled early), their final G' values were lower than that of the control (less than 200 Pa compared to ~300 Pa, $P < 0.05$). The final G' value of the milk with extremely methylated complexes (WPIA+2) was, for instance, only about 60 Pa. Extensive succinylation (WPIA-2) also seemed to be unfavourable to final G' (170 Pa). In the gels with complexes alone, modification of the complexes increased the G' values at pH 4.5 (apart for WPIA-2) while, in milk systems, only the WPIA-1 led to higher G' values at pH 4.5 than control milk. The absence of any similarity between the relationship between the final G' value of skim milk gels (Figure 30) or of the corresponding gels of complexes (Figure 29) and the pI of the complexes indicated that electrostatic interactions between the complexes (e.g., as micelle-bound complexes) do not account for the final firmness of the acid milk gels. Indeed, while the cancelation of repulsive interactions like electrostatic repulsion increases the chances for contact between the casein particles, attractive interactions or bonds are probably needed to actually build and sustain the gel network, and consequently to have a significant effect on the final G' of gels. In opposition to Roefs & Van Vliet (1990) who added NaCl to milk acidified at 2°C then warmed, the present results indicated that electrostatic attractions (between groups of opposite charges) do not contribute to the formation of acid milk gels. In their review,

Morand et al. (2011a) rather proposed that the roles of hydrophobic interactions and disulphide bonds should be evaluated using the present approach.

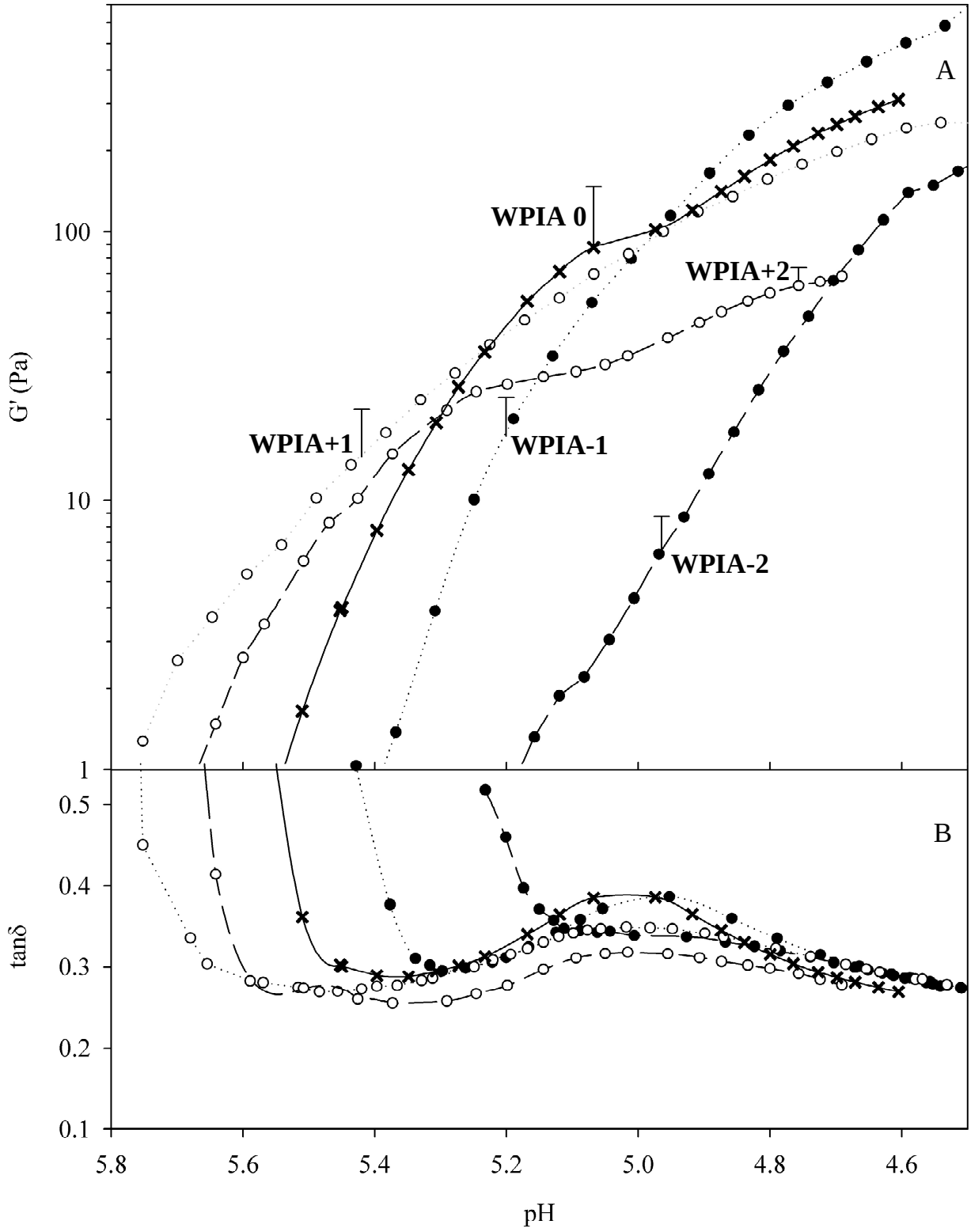

Figure 30 : Development of the elastic modulus (G' in Pa) (A) and loss tangent (tan δ (B) of reconstituted 50 g kg^{-1} skim milk systems containing 40 g kg^{-1} native phosphocaseinate (NPC) and 10 g kg^{-1} heat-induced whey protein complexes, during acidification by 18 g kg^{-1} GDL at 35°C

The final tan δ value of milk with modified complexes reached about 0.27 ± 0.00 and were slightly higher than that of the control (0.25, $P<0.05$). This result indicated that the chemical modifications induced somewhat more viscous-like milk acid gels, in agreement with the observations for the acidified suspensions of complexes. Moreover, the presence of casein micelles in the milk systems, as compared to those made of heat-induced complexes alone, led to more viscous-like behaviour of the acid gels, probably due to a less rigid structure of the micelle as compared to complexes.

Final gels at pH 4.5 were then characterised using large deformation rheology and penetrometry. The yield stress point of the gels (Figure 31A) indicates the difficulty in breaking protein strands, while the initial slope of the penetration force in the gel (stiffness, Figure 31B) indicates the rigidity of the gels. The results suggested two groups of samples, namely the gels with WPIA-1 complexes and the others. The yield stress point as well as the initial slope were significantly higher in acid gels including this complex ($P<0.05$ for the two parameters), than for the other samples. The results indicated the distinct behaviour of milk gels with WPIA-1 complexes (pI ranging 4.0 to 4.4, hatched zone). Considering the pI values, WPIA-1 was the closest to the natural complexes in heated milk (pI ~4.5, Jean et al., 2006). In fact, at pH 4.5, the methylated and control complexes were already positively charged, while the WPIA-2 complexes were still negatively charged and the WPIA-1 complexes bore almost no charge (Figure 26). At pH 4.5, both WPIA-1 and casein micelles were therefore close to neutrality, which could be favourable to the establishment of interactions between proteins.

Granulometric characterisation of the final gels confirmed the observation that WPIA-1 differed from the other samples. Confocal images of the acid gels are shown on Figure 31C. The granulometric curves showed a mean mode at 3.1 µm, representing the characteristic size of dark zones; this value was interpreted as the mean pore size of the gel. The PCA analysis showed that the gels with WPIA-1 complexes had a higher frequency of smaller pores than the other gels. The acid gel of milk systems containing complexes with almost the lowest surface charge at pH 4.5, due to a low level of succinylation, seemed to be more homogeneous due to a reduced pore size. These facts could explain why the milk gels with WPIA-1 complexes were the firmest at pH 4.5.

It is interesting to notice that, at pH 5.6, the milk samples with methylated complexes were the only systems that had already gelled. At pH 5.4, their G' values were still higher than that of the control. Unfortunately, their G' values at pH 4.5 were also undesirably low. However, methylation (or increased pI of the complexes) could therefore be a good way to obtain satisfactory acid milk gels with a high final pH (pH 5.4 *vs* pH 4.5).

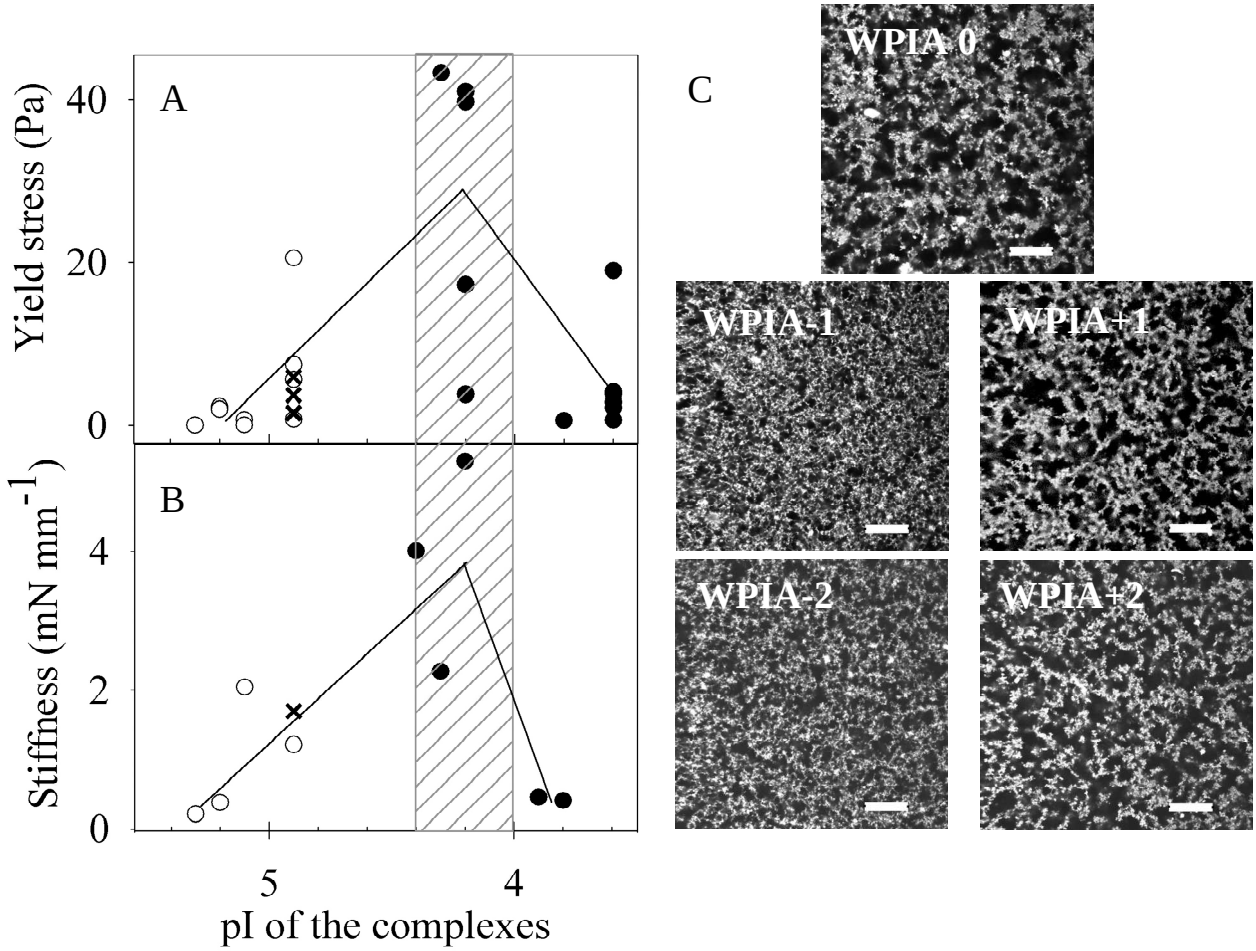

Figure 31 : Effects of modification of pI of complexes on rheological properties of the milk gels at pH 4.5. Panel A shows yield stress at large deformation (in Pa) while panel B shows stiffness after overnight holding at 4 °C, illustrated by the initial slope of the measured force versus depth of the cylinder during compression of the gel (in N mm-1). The crosses represent milk systems with the control complexes, the full circles are with succinylated complexes and the open circles with methylated complexes. The hatched zone corresponds to the firmer gels with complexes with pI value ranging between 4.0 and 4.4 (WPIA-1). Microstructure of the different milk systems at pH 4.5 was observed by confocal laser scanning microscope (C, scale bar 20 µm)

3.4. Surface charge of complexes at the gelation point of the two systems

The values of the electrophoretic mobility of the complexes at the pH of gelation of each type of systems (suspensions of complexes or milk systems) were estimated on Figure 26 (open triangles and open squares, respectively). The results clearly showed that the electrophoretic mobility of the complexes at the gel point was the same in the two systems ($P>0.01$). This result strongly suggests that the onset of acid gelation of the skim milk systems depended on the stability of the heat-induced whey protein complexes throughout acidification. Possibly, the casein micelles do not take part in the early destabilisation of heated milk, other than to bear the complexes on their surface and to add mass to the precipitating particles.

Interestingly, Figure 26 also showed that the different complexes did not initiate gelation at a constant value of their surface charge (electrophoretic mobility). At the gelation point, complexes with a high pI, for instance, had an electrophoretic mobility of -0.39 ±0.7 µm cm V^{-1} s^{-1}, whereas complexes with a low pI still exhibited an electrophoretic mobility of -0.76 ±0.7 µm cm V^{-1} s^{-1} and intermediate complexes (the control) -0.60 ±0.04 µm cm V^{-1} s^{-1}. Whatever the systems (milk or WPIA suspension) and the modification (succinylation or methylation), a reduction of 0.5 µm cm V^{-1} s^{-1} of the electrophoretic mobitity of the complexes was required for the onset of gelation. It therefore seemed that decreasing the pI of the complexes using succinylation allowed them to destabilize at a higher residual net charge. It is otherwise possible that succinylation favoured attractive hydrogen bonding more than methylation, since carboxyl groups are significant hydrogen bond exchangers. Alternatively, variation of the net charge of the complexes on gelation may result from experimental artefacts due to the transient ionic conditions applied during acidification, while the electrophoretic mobility measurements are made in equilibrium conditions.

4. Conclusions

It was possible to modify the pI of model heat-induced whey protein complexes with limited variation of their other physico-chemical properties. The introduction of such modified soluble complexes into model whey protein-free milk clearly show that the pI of the heat-induced complexes determines the onset of acid gelation of model heated milk systems. The near

neutralisation of their repulsive net charge seemed to provoke the destabilisation of the whole protein system, i.e., including the casein micelle. However, there were also indications that electrostatic repulsion/attraction is essential but not the only interaction that delays/initiates acid gelation. Also, the pI of the heat-induced complexes had no effect on the final G' value of the acid gels, probably because the building of the network clearly depends on constructive attractive interactions or bonds rather than on the only cancelation of repulsions.

Acknowledgements

The authors acknowledge Julien Jardin and Valérie Briard-Bion, UMR STLO INRA-AGROCAMPUS OUEST, for their expert help in mass spectroscopy analyses. This work was financially supported by Région Bretagne, under the grant ARED 4298. The MALLS equipment was funded by the French Research Agency (ANR), the European Union, Région Bretagne, Rennes Métropole and Conseil Général 35.

V.3. Résultats et discussion complémentaires

V.3.1. Mesure du rayon de gyration et de la masse moléculaire des complexes

Dans l'article 3, nous avons rapporté une augmentation du diamètre hydrodynamique (D_h) des complexes succinylés (Figure 21), accompagnée d'une perte légère perte de leur structure (rupture de feuillets β intramoléculaire). Les valeurs du rayon de gyration (R_g) et de la masse moléculaire (M_w) des complexes, obtenus par SEC-MALLS, n'étaient pas significativement différentes d'un complexe modifié à l'autre et étaient respectivement de 28 ± 4 nm et 6.106 ± 2.106 g mol-1(Figure 32), En revanche, les complexes extrêmement succinylés (pI > 3.5), n'ont pas pu être analysés en SEC-MALLS du fait de leur trop grande taille (D_h > 200 nm),. Ces résultats suggèrent le gonflement des complexes suite à la succinylation plutôt que leur suraggrégation, ce qui confirme l'hypothèse émise dans l'article 3 d'une augmentation de la voluminosité des complexes.

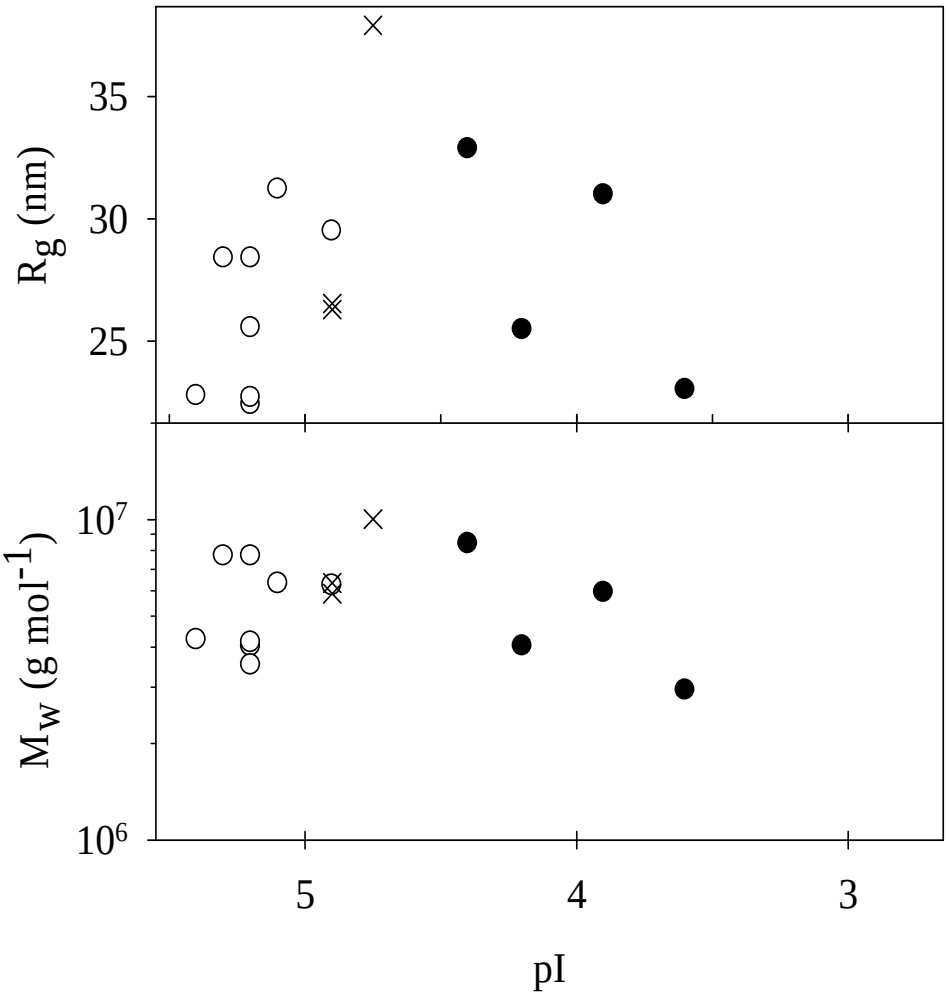

Figure 32 : Rayon de gyration (R_g) (A) et masse moléculaire (M_w) (B) des complexes thermo-induits en fonction de leur point isoélectrique (pI). Complexes témoins (×), complexes succinylés (●) et complexes méthylés (○)

V.3.2. Comparaison des mesures de l'hydrophobie des complexes obtenues par les méthodes de fixation de l'ANS ou du Prodan

En continuité avec la partie 1, les premières mesures de l'hydrophobie des complexes thermo-induits succinylés et méthylés ont été réalisées, dans un premier temps, par la méthode de fixation de l'ANS (PSH_{ANS}). Les résultats, illustrés par la Figure 33A, montraient une diminution du PSH_{ANS} avec la diminution du pI des complexes. Or, la charge négative des complexes succinylés a été augmentée par la modification, et la sonde ANS étant elle-même chargée négativement, les répulsions électrostatiques complexes-ANS pourraient inhiber leurs interactions hydrophobes. Alizadeh-Pasdar & Li-Chan (2000) avaient observé la dépendance de la mesure de l'hydrophobie des protéines à la charge des protéines et de la sonde utilisée. La diminution de l'index PSH par cette méthode pourrait donc refléter les modifications de charge de complexes.

Afin de vérifier si l'hydrophobie des complexes a été modifiée par la succinylation, nous avons choisi de poursuivre les mesures par la méthode de fixation du Prodan. Cette sonde étant non-ionique, son interaction avec les complexes ne sera pas perturbée par la modification de leur charge. Les résultats sont illustrés par la Figure 33B. Avec cette méthode, on constate que l'hydrophobie des mêmes complexes que sur la Figure 33A est constante quel que soit leur pI.

Dans la partie 1, nous avions observé une diminution du pI des complexes riches en caséine-κ sans que leur hydrophobie, par la méthode de la fixation de l'ANS, ne soit modifiée. Les mesures avec la méthode du Prodan n'ont pas été réalisées.

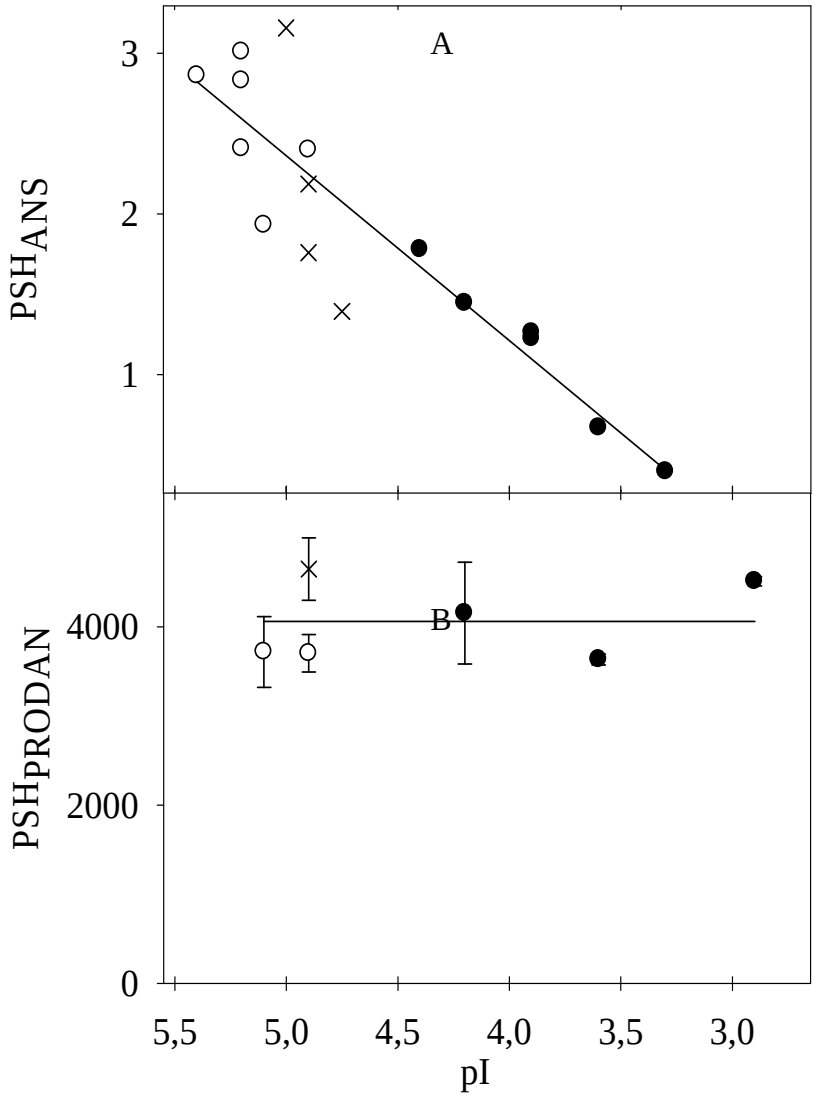

Figure 33 : Hydrophobie de surface des complexes thermo-induits déterminée par la méthode de fixation de l'ANS (PSH_{ANS})(A) et par la méthode de fixation du Prodan (PSH_{Prodan}) (B) en fonction de leur point isoélectrique (pI)

V.4. Bilan de la partie 2

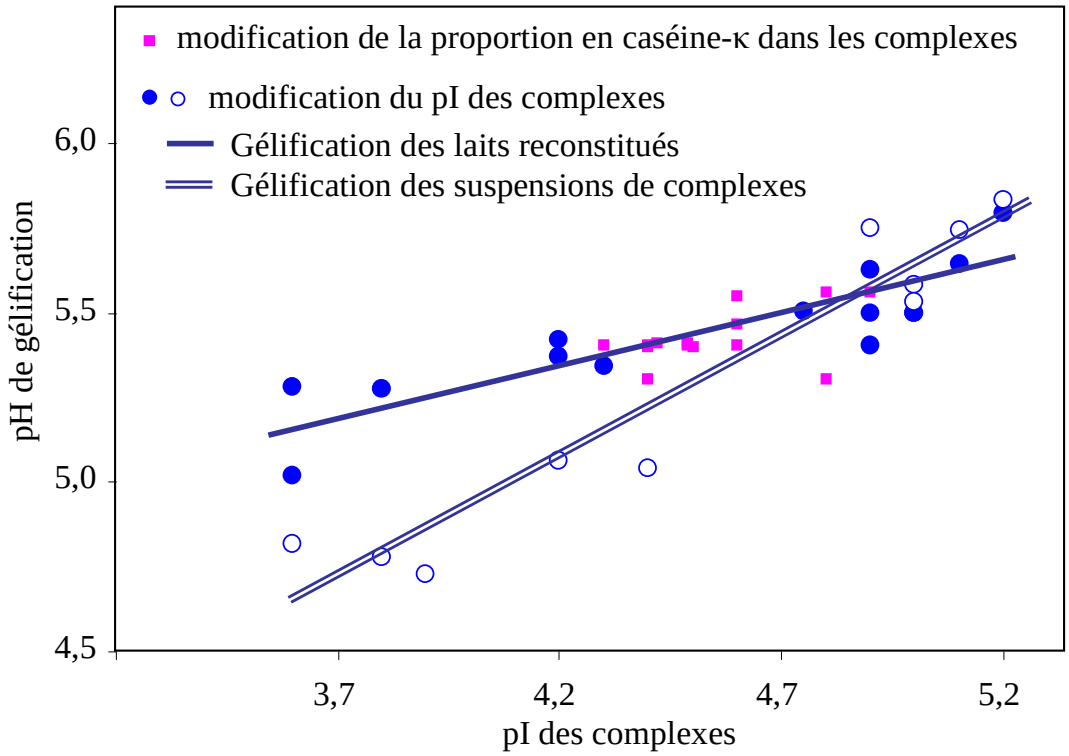

Figure 34 : pH de gélification des systèmes laitiers (suspensions de complexes ou laits reconstitués avec les complexes) en fonction du point isoélectrique (pI) des complexes thermo-induits

Dans cette partie de résultats, nous avons considérablement élargi la gamme de variation du pI des complexes thermo-induits par rapport au chapitre précédent (■, Figure 34). Grâce aux réactions de méthylation et de succinylation, le pI des complexes a été modifié entre 3.8 et 5.3, sans que les autres propriétés physico-chimiques ne soient significativement affectées. La diminution du pI des complexes entraîne la diminution du pH de gélification des systèmes laitiers (suspensions de complexes et laits reconstitués avec les complexes, Figure 34). La gélification des systèmes nécessite donc la réduction des répulsions électrostatiques entre les complexes. Pour un complexe de pI donné, les pH de gélification des suspensions de complexes et des laits reconstitués correspondants ne sont pas très éloignés. Ce résultat met en évidence que malgré leur faible proportion dans les laits reconstitués (20% w/w des protéines), les complexes thermo-induits imposent leurs propriétés à la micelle de caséines. Ce phénomène peut être expliqué par la fonctionnalisation de la surface de la micelle par les complexes au

cours de l'acidification. Dans le cas général du lait, cette fonctionnalisation intervient également au cours du traitement thermique (formation de complexes micellaires). Dans ce chapitre, nous avons également montré qu'il persiste une charge négative résiduelle sur les complexes au pH de gélification des systèmes. Ce résultat met en évidence la contribution d'autres interactions attractives dans la déstabilisation des systèmes.

Chapitre VI.

Résultats et discussion – Partie 3

Rôle de l'hydrophobie des complexes thermo-induits sur la déstabilisation par acidification et la construction de systèmes laitiers

VI.1. Introduction

D'après les résultats de la partie précédente, nous avions conclu que la neutralisation des répulsions électrostatiques au cours de l'acidification contribue à la déstabilisation du lait, mais que d'autres interactions attractives doivent participer à la formation du gel acide. D'après l'étude bibliographique (§II.6.2), les interactions hydrophobes sont nos premières candidates. Nous souhaitons donc tester le rôle de l'hydrophobie de surface des complexes thermo-induits sur la déstabilisation du lait et la construction du gel acide. Selon la stratégie du projet, l'hydrophobie des complexes a été modifiée par des réactions d'acylation de chaines carbonées de longueurs croissantes (entre 2 et 6 carbones) sur les lysines, apportant donc une hydrophobie croissante.

Cette troisième et dernière partie de résultats a fait l'objet un stage de 6 mois de master 2, réalisé par Assiba Dekkari, étudiante à l'université Paul Cezanne de Marseille. Les travaux réalisés ont été présentés lors d'une conférence au cours du congrès « les rencontres du grand ouest », RBPGO 2011 à Rennes, France en juin 2011. Un article a récemment été soumis pour publication dans le journal *International Dairy Journal*. L'intégralité de l'article est proposée dans ce chapitre pour présenter les résultats obtenus et leur discussion.

VI.2. Article 4

Increasing the hydrophobicity of the heat-induced whey protein complexes improves the acid gelation of skim milk

Marion MORAND[ab], Assiba DEKKARI[ab], Fanny GUYOMARC'H[ab], Marie-Hélène FAMELART[ab,*]

ABSTRACT

The formation of whey protein complexes during heating is known to enhance the acid gelation of milk. Hydrophobic interactions have been suggested to play an important role in the acid gelation of milk or milk protein ingredients. To investigate this, the surface hydrophobicity of model heat-induced whey protein complexes was modified using acylation with various carbon chain lengths. It was checked that the size and thiol/disulfide distribution of the complexes were unaffected, and that the change of their apparent isoelectric point could be restricted within 0.5 pH unit. These complexes were added to whey protein-free skim milk systems and the resulting acid-gelation behaviour of the milk samples was measured. The results showed that increasing the hydrophobicity of the heat-induced whey protein complexes significantly increased the pH of gelation of the milk samples and strongly affected the final properties of the acid gels. This demonstrated both the importance of the heat-induced complexes and the relevance of hydrophobic interactions in the acid-induced gelation of preheated milk.

1. Introduction

The heat-treatment of milk at 85-95 °C for several minutes has long been reported to increase both the pH of gelation and firmness of acid milk gels. These changes have been attributed to the formation of heat-induced whey protein/κ-casein complexes in the milk. In their review, Donato & Guyomarc'h (2009) have proposed that the heat-induced whey

protein/κ-casein complexes alter the acid gelation behaviour of the casein micelles through interacting with their surface on heat-treatment (micelle-bound complexes) or early on acidification (soluble complexes), thus modifying colloidal interactions such as electrostatic repulsion or hydrophobic attraction. Heat-denatured whey protein ingredients can be substituted to the whey protein/κ-casein complexes (Morand et al., 2011b; O'Kennedy & Kelly, 2000; Schorsch et al., 2001) and may be used as vectors to modify the acid gelation behaviour of skim milk (Morand et al., 2011a). In line with this hypothesis, Morand et al. (2012) chemically modified the apparent isoelectric point (pI) of heat-induced whey protein complexes in order to assess the role of electrostatic repulsion on the acid gelation of skim milk. The results showed that the increase of the apparent pI of the complexes significantly increased the pH of gelation of milk. It was concluded that neutralisation of the repulsive net charges of the complexes on acidification contributed to the destabilisation of heated milk. However, modification of the pI did not induce significant changes in the texture of the final gels. It was therefore suggested that attractive interactions other than electrostatic are responsible for the actual building of the network. The present paper reports on how changes in the surface hydrophobicity of heat-induced whey protein complexes affect the acid gelation behaviour of skim milk. The surface hydrophobicity of model complexes was increased using acylation with a range of carbon chain lengths. The modified complexes were characterised then introduced in whey protein-free milk, whose acid gelation behaviour was measured.

2. Materials and Methods

2.1. Materials

Native micellar casein (NMC, 870 g kg^{-1} of proteins on dry basis, whey protein level: 5 % of the total protein) was prepared as described by Schuck et al. (1994). Briefly, raw milk was skimmed at ~50°C then microfiltered at that temperature through 0.1 µm cut-off membrane, diafiltered, evapo-concentrated and spray-dried. The microfiltration permeate, containing the native whey proteins, was concentrated by ultrafiltration at ~50°C, diafiltered and freeze-dried to yield a native whey protein isolate (WPI, 970 g kg^{-1} of proteins on dry basis). The protein composition of the WPI as given by reverse phase-high performance liquid chromatography

(RP-HPLC) was, in % of the total area: ~80% whey proteins, ~10% β-casein and minor amounts of κ-, α_{s1}- and α_{s2}-caseins. The milk ultrafiltration permeate (MUF) resulting from UF-concentration of the whey protein fraction was collected and stored at 5°C after addition of 0.2 g L^{-1} sodium azide. All other reagents were of analytical grade.

2.2. Preparation of the heat-induced complexes

A model whey protein complex (WPIA 0) was prepared as described by Vasbinder et al. (2004). Briefly, a 90 g kg^{-1} solution of WPI in deionised water with 0.2 g kg^{-1} NaN_3 was adjusted at pH 7.5 then heated at 68.5°C for 2 h. The heat-induced complexes were then diluted to ~30 g kg^{-1} with deionised water and subsequently acylated using anhydrides derivatives of various chain lengths as described by Gerbanowski et al. (1999). Acylations were performed at pH 8 with acetic, butanoic, hexanoic or succinic anhydride at concentrations ranging from 1 to 3 anhydride molar equivalents per lysine for 2 h. The reactions yielded the modified complexes WPIA-C2, -C4, -C6 or -C4$^-$, respectively. The acylation reaction involves the chemical conversion of lysine residues of the complexes. It results in the elimination of the basic group of the lysine residue (Figure 35A). Depending of the length of the anhydride carbon chain, the hydrophobicity of the complexes can be modulated. Acylation with succinic anhydrides results in the replacement of the basic group of the lysine by the carboxyl group of one aliphatic acid (Figure 35B).

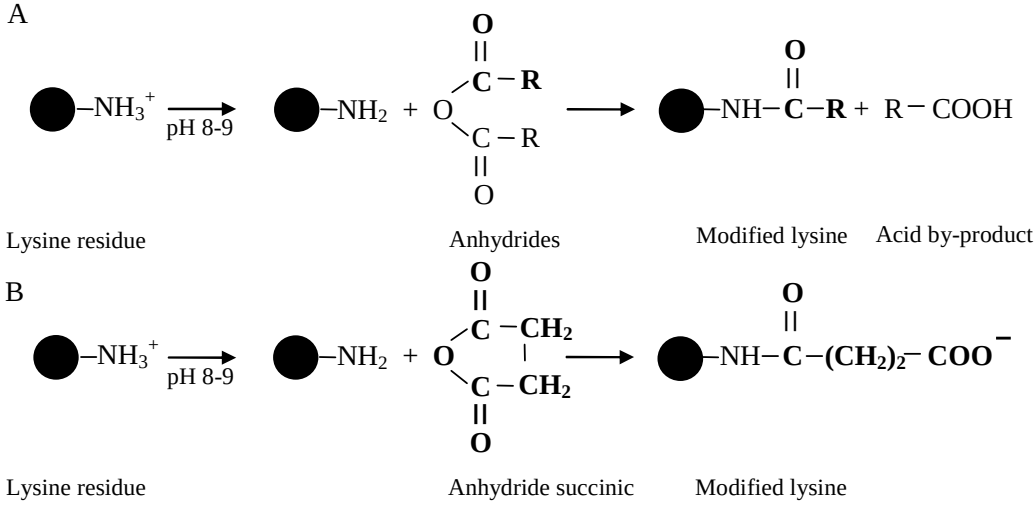

Figure 35 : Schematic diagram of the acylation (A) and succinylation (B) reactions used to modify the surface hydrophobicity of whey protein heat-induced complexes. Three anhydrides were used for acylation (A): acetic (R = CH_3), butanoic (R = $(CH_2)_2CH_3$ and hexanoic (R = $(CH_2)_4CH_3$)

After the reaction period, the solutions were extensively dialysed (6-8 kDa, Medicell International Ltd., London, UK) against deionised water adjusted at pH 7.5 and 0.2 g kg^{-1} NaN_3 as preservative, to eliminate by-products and residual reactants. The final total protein content of the complexes solutions was determined using absorbance at 280 nm and an extinction coefficient value of 1.046 L $g^{-1}.cm^{-1}$ for the WPIA (Morand et al., 2011b).

2.3. Evaluation of the degree of modification

The degree of acylation of the samples was assayed using ortho-phthaldialehyde (OPA) as described previously by Morand et al. (2012). In presence of 2-dimethylaminoethanothiol hydrochloride (DMA), OPA reacts with the free primary amino groups of proteins and yields alkyl-iso-indole derivates that absorb light at 340 nm. The degree of modification was given as a percentage of the total primary amino groups found in the control model complex WPIA 0. Each determination was performed twice on an Uvikon 922 spectrophotometer (Kontron, Paris, France).

2.4. Physico-chemical properties of the complexes

The physico-chemical properties of the WPIA complexes, namely their surface hydrophobicity, their apparent isoelectric point, their size and their contents in surface and total free thiol groups and in total cysteine residues were measured using the routine methods described by Morand et al (2012; 2011b). Briefly, the protein surface hydrophobicity index (PSH) was determined in phosphate buffer at pH 7.0 using a constant concentration of the non-ionic 6-propionyl-2-(N,N-dimethylamino)-naphthalene probe (PRODAN, Molecular Probes). The PSH was taken as the initial slope of the fluorescence versus the protein concentration. The apparent isoelectric point (pI) of the heat-induced whey protein complexes was determined in MUF adjusted at various pH ranging ~2 to 6.7 and corrected for ionic calcium. The pI was taken as the interpolation to 0 of the experimental measurement of the electrophoretic mobility of the complexes (in µm cm V^{-1} s^{-1}) using a Zetasizer nanoZS Malvern (Malvern Instruments, Orsay, France). The hydrodynamic diameter D_h of the complexes was determined at 20°C in MUF using dynamic light scattering at a fixed angle of 173° on the same Zetasizer equipment. The accessible thiol groups (surface SH), the total free thiol groups (SH) and the total cysteine

residues (S) of the WPIA complexes were assayed using the 5,5'-dithio-bis-2-nitrobenzoïc acid (DTNB) reagent method (Ellman, 1959) on aliquots respectively diluted in non-dissociating Tris buffer at pH 7.4, in dissociating urea-citrate-sodium dodecyl sulphate-Tris buffer at pH 7.4 or in dissociating buffer in presence of β-mercaptoethanol (Morand et al., 2011b). The TNB^{2-} product was assayed using absorption at 412 nm.

2.5. Acid gelation behaviour of the WPIA

First, the whey protein complexes were extensively dialysed (6-8 kDa MWCO, Medicell International Ltd., London, UK) at 4°C against commercial ultra-high temperature (UHT) half-fat milk in order to replace their solvent phase with milk permeate. The resulting protein concentration was determined spectrophotometrically, then each WPIA suspension was standardized at 20 g kg^{-1} total protein using MUF. The suspensions were then equilibrated at 35°C prior to acid gelation using 17 g kg^{-1} glucono-δ-lactone (GDL). The formation of the gels was monitored using an AR2000 rheometer (TA Instruments, Guyancourt, France) equipped with a cone-plane geometry and using the oscillatory mode at a frequency of 1 Hz and a strain of 0.1%. The pH was recorded simultaneously (Morand et al., 2012). Gelation time was defined as the moment when G' > 1 Pa.

2.6. Acid gelation behaviour of the reconstituted heated milk systems

A mother suspension of NMC was reconstituted at 65 g kg^{-1} in MUF with 0.2 g kg^{-1} NaN_3 at 40°C and stored overnight at 4°C. The NMC suspension, dialysed WPIA complexes and MUF were eventually mixed as to reach a total protein content of 50 g kg^{-1} and a NMC to complexes ratio of 80:20 w/w. The resulting systems were regarded as heated skim milk models with heat-induced soluble whey protein complexes. The heated milk systems were equilibrated at 25°C and labelled using 0.2 g kg^{-1} rhodamine B isothiocyanate solution (85 g L^{-1} RITC in dimethylsulfoxyde, Sigma-Aldrich, St Quentin Fallavier, France) prior to acid gelation. The formation of the acid gels was induced by addition of 19 g kg^{-1} GDL and was monitored at 25°C using the same method as for the WPIA suspensions (section 2.5). Preliminary experiments have showed that an acidification temperature of 35°C as used in Morand et al. (2012; 2011b) induced contraction and whey separation of the acid gels involving the modified

complexes. For that reason, the lower incubation temperature of 25°C was chosen to minimise slippering of the gel against the geometry. The gels at pH 4.5 were eventually subjected to a stress sweep test from 1.10^{-3} to 1 000 Pa in log increment. The yield stress (in Pa) was defined as the point when the G' started to decrease. Each milk system was prepared twice, and each preparation was analysed twice. In complement, compression tests were performed at 4°C on 10-mL aliquots of milk sample incubated separately at 25°C until pH 4.5 then kept overnight at 4°C (4501 INSTRON, Instron SA, Guyancourt, France) using a 10 N sensor and a 11 mm-diameter, 45 mm-height plastic cylinder geometry. The apparent stiffness of the gels (in N mm^{-1}) was defined as the initial slope of the force versus depth on penetration of the cylinder in the gel at a speed of 120 mm min^{-1}. Five aliquots were measured by this method.

Immediately after GDL dispersion, one drop of RITC-labelled milk sample was deposited on a sealed conclave slide and incubated at 25°C until pH 4.5. The slide was then imaged at 543 nm using a TE2000-E Nikon C1i inverted confocal laser scanning microscope (CLSM, Nikon, Champigny-sur-Marne, France). All the settings were as in Morand et al. (2012). Each image was digitized in grey levels as a 512 x 512 pixel matrix (127.3 x 127.3 μm^2). A grey level granulometric method from mathematical morphology was applied as described previously by Devaux et al. (2008). The method consists in sequentially applying image filters (defined by squares of side lengths ranging 0.75-50 µm) that delete objects smaller than the filter at each step. By considering the derivative of the sum of grey levels obtained at each step, one obtains a curve of the distribution of the sizes of grey regions in the image. Morphological closing was used, making dark regions to disappear, and is therefore adapted to measure the 2D size of pores in the gels. A principal component analysis (PCA) was performed on these curves for all images, using the R package (R 2.9.2. Foundation for Statistical Computing).

3. Results and discussion

3.1. Rate of acylation of the complexes and changes in their surface hydrophobicity and pI

The OPA assay showed that 13 to 100% of the ε-amino groups found in control WPIA were converted into carbon chains in the modified complexes WPIA-C2, C4, $C4^-$ and C6 as a result

of acylation (Figure 36). The rate of acylation increased with increasing the molar ratio of anhydride to whey protein (not shown). Figure 36A shows the surface hydrophobicity (PSH) of the complexes as a function of the type of anhydride used and of the acylation rate. As expected, the PSH of the complexes increased linearly with the acylation rate of the WPIA-C2, C4 and C6 complexes ($P < 0.02$, 0.01 and 0.01, respectively). Furthermore, the longer the grafted carbon chain, the higher the slope of the linear regression. The PSH of these modified complexes varied between ~5000 and 16000 and were significantly higher than the WPIA-0 controls ($P < 0.002$). On the other hand, the modification of the WPIA-0 complexes with succinic anhydride yielded WPIA-C4$^-$ complexes with similar PSH values than those of the WPIA-0 complexes ($P > 0.7$). This result was in agreement with Morand et al. (2012).

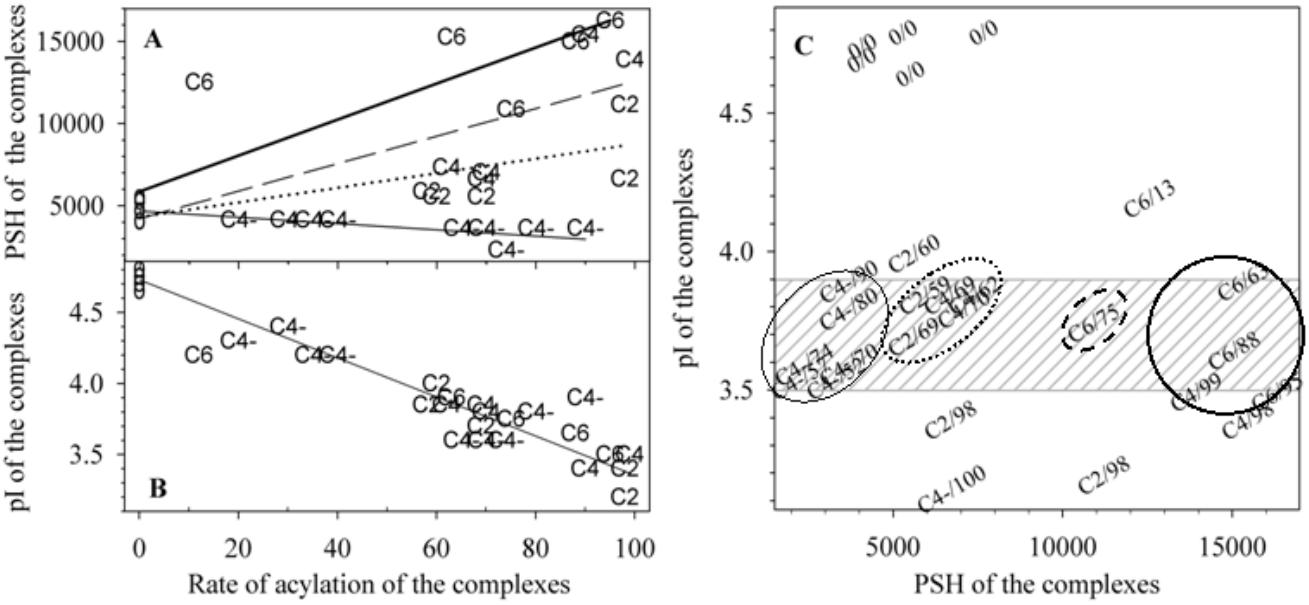

Figure 36 : (A) Modification of the surface hydrophobicity (PSH) and (B) of the apparent isoelectric point (pI) as a function of the rate of acylation of the complexes with acetic anhydride (C2), butanoic anhydride (C4), hexanoic anhydride (C6) and succinic acid (C4$^-$). (C) pI value of the complexes as a function of their PSH. Data points are labelled as "carbon chain/acylation rate". A selection of 4 groups of samples with a pI of 3.7 ± 0.2 (hatched zone) and similar PSH values are shown: PSH ~3200 (—), ~6500 (·····), ~11000 (– – –), ~15000 (▬). Sample points are located in the centre of the labels and lines are for linear regressions

Since the acylation reaction transforms basic amine groups into either neutral carbon chains (for C2, C4, C6) or carboxylated carbon chains (for C4$^-$), it is expected that it reduced the apparent isoelectric point (pI) of the complexes. Figure 36B shows the measured pI values of the complexes as a function of the reaction type and acylation rate. In agreement with previous reports (Morand et al., 2012; 2011b), the pI of the control complexes was ~4.8 and decreased

linearly down to below 3.5 as the rate of acylation of the complexes increased (≥95% modification; $P < 0.01$).

Alting, et al. (2000) or Morand et al (2012; 2011b) have shown that the electrostatic properties of the whey protein complexes significantly affect their acid gelation behaviour. In order to study the only effect of the surface hydrophobicity on acid gelation, 4 groups of samples were therefore selected that had pI values within 3.7 ± 0.2 (hatched zone, Figure 36C) but different PSH values.

3.2. Other physico-chemical properties of the complexes

Changes in the size and thiol/disulfide distribution of the heat-induced whey protein complexes are likely to affect acid gelation (Donato & Guyomarc'h, 2009; Morand et al., 2011a). Therefore, these properties were measured for all the control and modified samples in order to anticipate possible bias. The mean hydrodynamic diameters, D_h of the selected WPIA-0, WPIA-C2, WPIA-C4 and WPIA-C6 complexes were about 90 nm ± 13 nm. The mean D_h of WPIA-C4$^-$ samples was significantly different from the other samples (D_h = 120 ± 12 nm, $P < 0.01$). This size increase of the succinylated complexes has previously been observed for rates higher than 70-94% (Alting et al., 2002; Lieske, 1999; Morand et al., 2012). According to these authors, electrostatic repulsions between protein chains may increase in the particles, which would increase their voluminosity. However the secondary structure of the complexes is hardly modified during succinylation and an increase of D_h up to 130 nm has no effect on the acid gelation of milk (Morand et al., 2012).

The total cysteine content of all samples was ~152 ± 30 µmol g^{-1} total protein and, as expected, did not show significant differences between samples ($P > 0.4$). The total free thiol content was found to be about 11 ± 2 and 5 ± 3 µmol g^{-1} total protein for the WPIA-0 and the modified complexes, respectively. It is possible that the binding of the carbon chains occurred to some extent on the sulfhydryl groups rather than on the primary amines, as can occur during succinylation (Aitken & Learmonth, 2002a). On the other hand, the free SH represented less than 7% of the total cysteines in all the samples. Also, surface SH were negligible in all the complexes (< 3 µmol g^{-1}). These results indicate that the majority of the cysteines were involved in disulfide bridges.

In conclusion, neither the size or the thiol/disulfide composition of the modified complexes was considered different to those of the control complexes, with respect to the acid gelation behaviour of the complexes.

3.3. Effect of the surface hydrophobicity of heat-induced complexes on acid gelation

3.3.1. Acid gelation of suspensions of complexes alone at 35°C

The influence of the surface hydrophobicity was first studied on the acid gelation of suspensions of complexes alone. Figure 37 shows the mean profiles of the changes in elastic modulus G' and in tanδ throughout acidification of the 4 groups of samples having PSH values ranging from 3200 to 15000 and a constant pI of 3.7 ± 0.2. Some samples with high PSH values expelled whey at the bottom of the gel on acidification, so that G' could not be reliably measured at pH values below ~5.0. For that reason, the data were analysed with respect to the maximal value of G' obtained during acidification (G'_{max}) rather than G' at pH 4.5 (usually taken as final G'). Figure 38 shows the experimental values of the pH of gelation and of G'_{max} as a function of the PSH of the heat-induced complexes. The results showed that both the pH of gelation and the G'_{max} of the acid gels increased linearly from 4.7 to 5.9, and from 45 to 835 Pa respectively, as the PSH of the complexes increased from 3200 to 15000 ($P < 0.01$). At the pH of gelation, the electrophoretic mobility of complexes having PSH values increasing from 3200 to 15000 was found to increase in absolute value from -0.7 to -1.0 µm cm V^{-1} s^{-1} (not shown). This observation showed that the particles could interact in spite of significant residual negative charge, probably thank to their increased hydrophobic attractivity. When modifying only the net charge and pI of the complexes, Morand et al (2012) found that a maximum net charge of -0.8 µm cm V^{-1} s^{-1} could be overcome with complexes of similar PSH. On the other hand, changes in the tanδ of the systems with pH did not differ across the samples. Tanδ decreased rapidly on gelation then tended towards the similar final value of ~0.18 ± 0.02 ($P < 0.1$ – Figure 37B).

These results indicated that complexes exposing more hydrophobicity gave earlier and firmer acid gels, up to showing propensity to contraction and whey separation. Similar tanδ

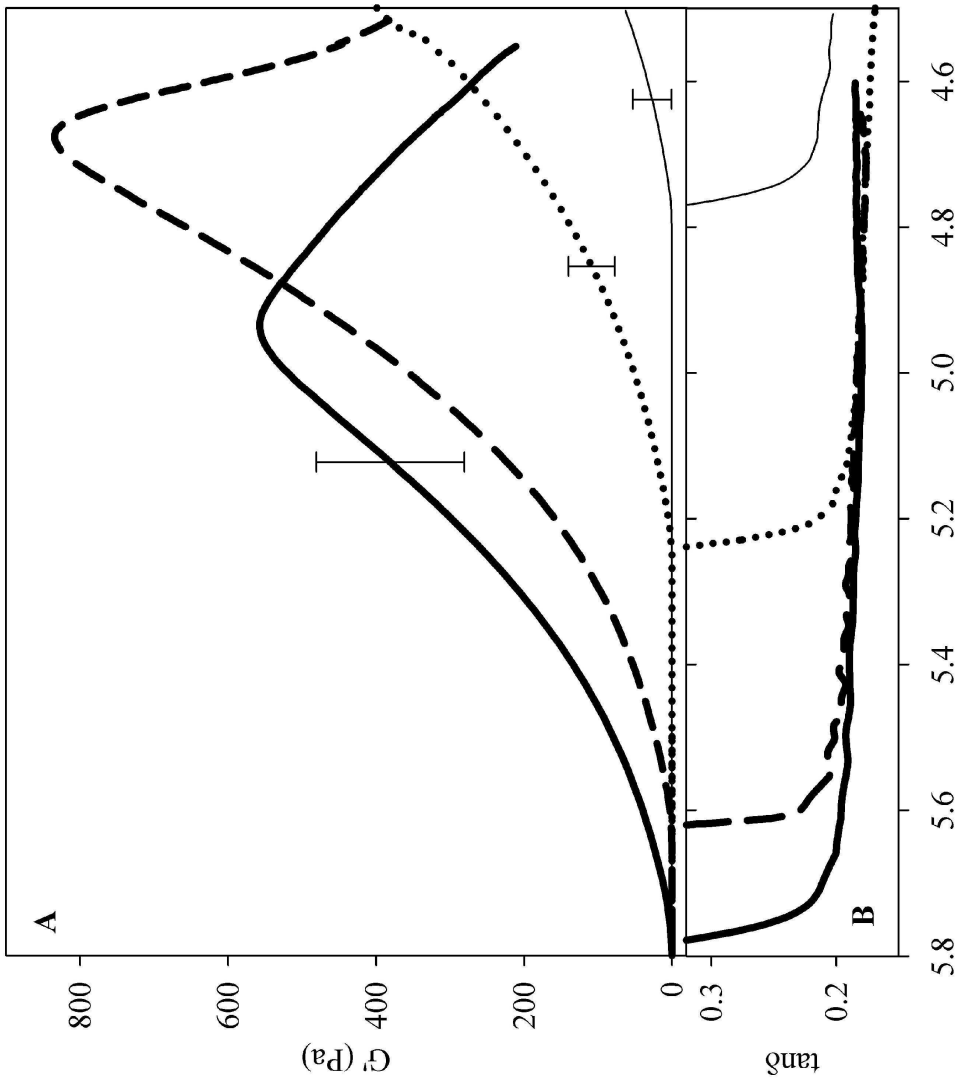

Figure 37 : (A) Development of the elastic modulus, G' (in Pa) and (B) of the loss tangent, tanδ, of suspensions of heat-induced complexes adjusted at 20 g kg-1 in milk ultrafiltrate, in the course of acidification by 17 g kg-1 GDL at 35°C. The complexes had a constant isoelectric point of ~3.7 and surface hydrophobicity values of PSH ~3200 (—), ~6500 (·····), ~11000 (– – –), ~15000 (▬)

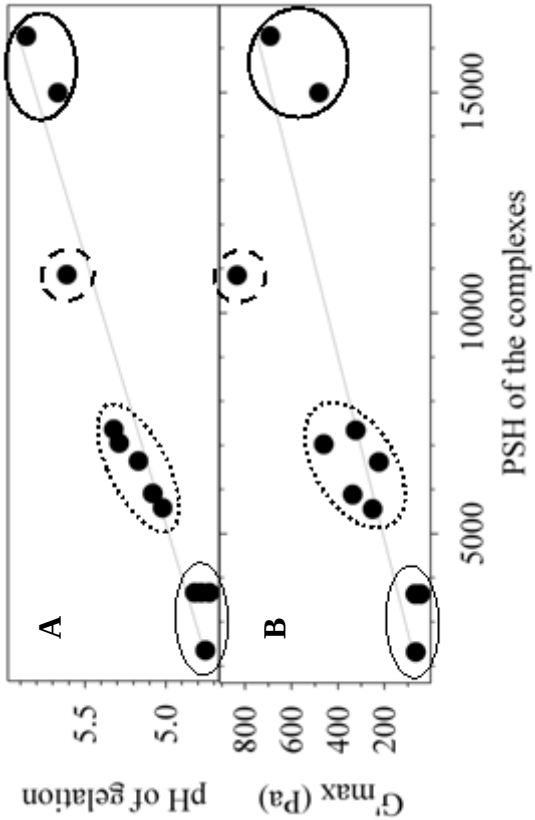

Figure 38 : pH of gelation (A) and maximal elastic modulus, G' (B) of acid gels obtained with suspensions of heat-induced complexes having a constant isoelectric point of ~3.7 and surface hydrophobicity values ~3200 (—), ~6500 (·····), ~11000 (▬ ▬ ▬), ~1500

indicated that the mechanical structure of the gel was reinforced by the increase in PSH of the complexes, rather than modified e.g. in the nature and/or energy of the bonds involved. These results were in agreement with Roefs & van Vliet (1990) who found that the elastic modulus of acid dairy gels at pH 4.6 increased with the incubation temperature, while the values of tanδ hardly differed. They respectively inferred from these observations that hydrophobic interactions were important to the formation of acid gels and that favouring hydrophobic attraction results in the increase of the number of bonds established in the gel. This proposition fits well to the present model of chemically modified heat-induced complexes, which probably have an increased number of hydrophobic sites on their surface.

3.3.2. Acid gelation of the recombined model milk system at 25°C

The 4 groups of modified complexes with PSH values ranging from 3200 to 15000 and a pI value of ~3.7 were introduced in whey protein free milk. As presented previously for the complexes alone, Figure 39 shows the mean profiles of the elastic modulus G' and of tanδ with time throughout acidification of the 4 groups of samples. Again, samples with high PSH values sometimes exhibited whey separation at pH values below ~5.0 so that their G' could not always be reliably measured. Figure 40 shows the experimental values of the pH of gelation and of G'_{max} as a function of the PSH of the heat-induced complexes. Figure 39A and Figure 40A show that increasing the PSH of the complexes from about 3200 to 8000 induced a linear increase of the pH of gelation of the reconstituted milk systems from 4.9 to 6.1 ($P < 0.01$). However, as the PSH value exceeded 8000, the pH of gelation of the milk samples did not increase anymore and the G' decreased. In fact, due to the initially rapid drop in pH induced by the GDL, the systems with complexes at high PSH value probably started to gel before the first value of G' was recorded at about pH ~6.2. Therefore, changes in the pH of gelation of the milk samples can not be observed above that value.

Figure 39A and Figure 40B show that as the PSH of the complexes increased from 3200 to ~8000, the G'_{max} of the acid milk gels increased significantly ($P < 0.05$; $dG'/dPSH = 0.368$ Pa) from 80 to 2100 ± 500 Pa. For complexes of PSH values higher than ~8000, expelled whey and visible fractures in the gel led to a decrease in G'_{max} values. Therefore, increasing the PSH value of the heat-induced complexes from 2000 to 8000 had significantly increased both the

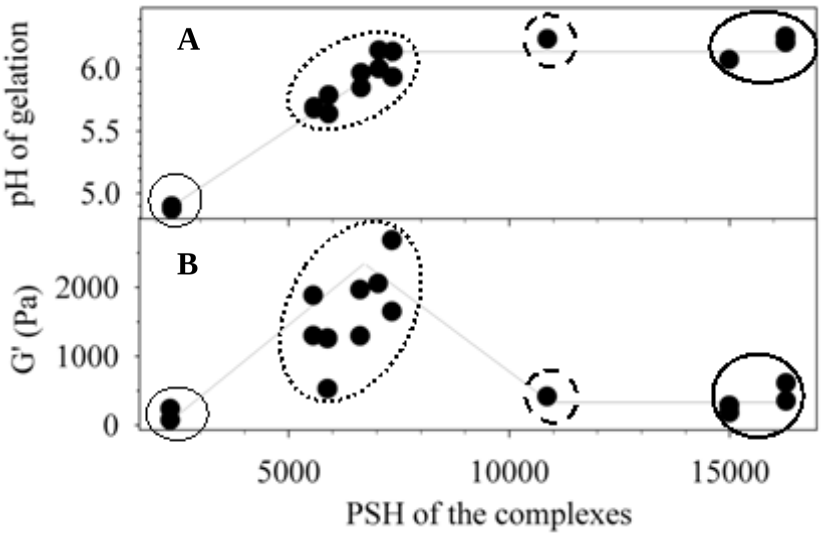

Figure 40 : pH of gelation (A) and maximal elastic modulus, G' (B) of acid gels obtained from model milk systems containing 40 g kg-1 casein micelles and 10 g kg-1 heat-induced complexes. Heat-induced complexes belong to 4 groups with increasing surface hydrophobicity (PSH) and with a fixed isoelectric point at 3.7

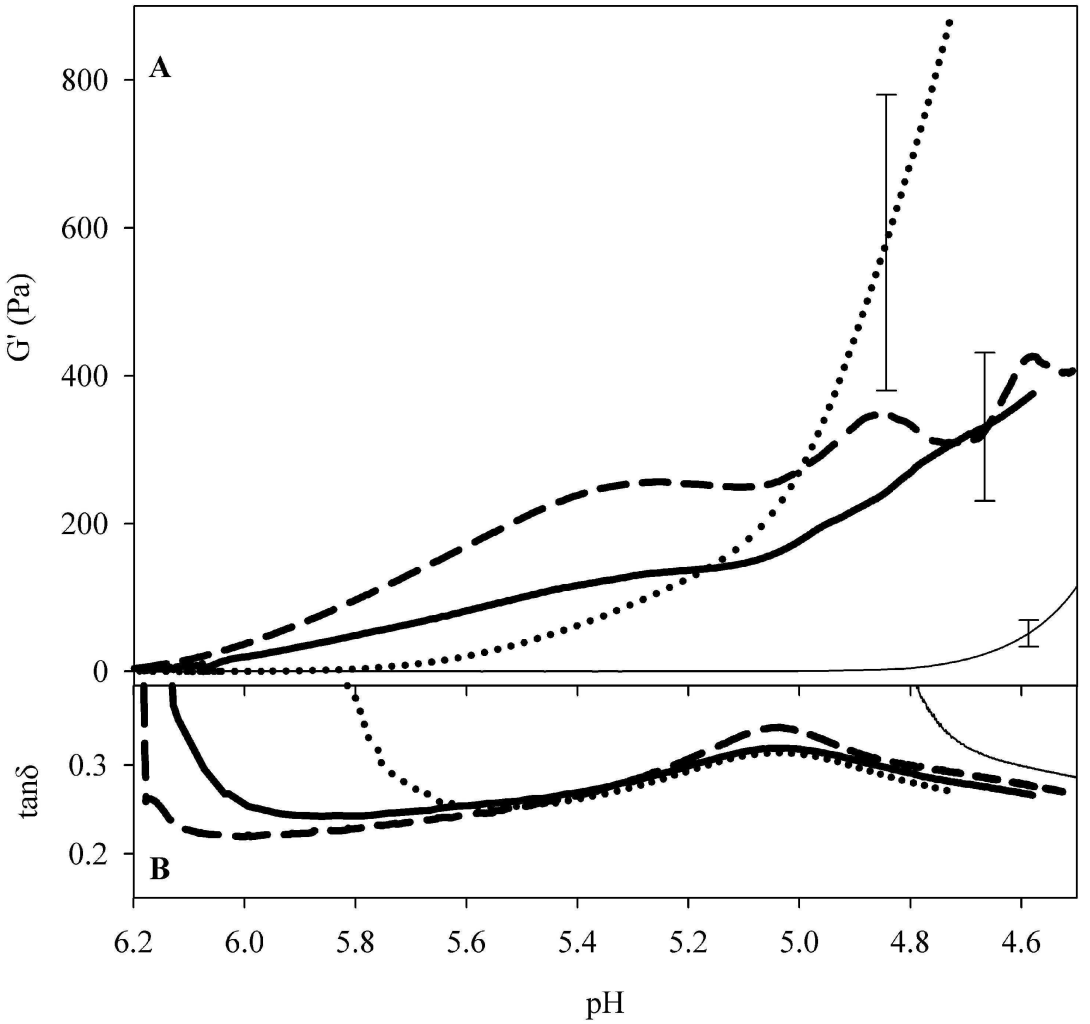

Figure 39 : Development of the elastic modulus, G' (A) and loss tangent, tanδ (B) of suspensions of reconstituted 50 g kg^{-1} skim milk systems containing 40 g kg^{-1} casein micelles and 10 g kg^{-1} heat-induced complexes, in the course of acidification by 19 g kg^{-1} GDL at 25°C. The complexes had a constant isoelectric point of ~3.7 and surface hydrophobicity values ~3200 (—), ~6500 (·····), ~11000 (– – –), ~15000 (▬)

gel strength and the pH of gelation. Figure 39B shows that in all the system studied except for WPIA-C4$^-$ milk sample (with PSH ~ 3200), tanδ decreased abruptly below 1 on gel formation, showed a local maximum at pH ~5.05 then decreased again to tend towards 0.24-0.30 at pH 4.5. This pattern is typical of acidified heated milk (Lucey & Singh, 1998). WPIA-C4$^-$ milk sample gelled at lower pH values than 5.0 and therefore only showed a decrease towards tanδ ~0.30. As in gels of complexes alone, similar tanδ values were measured in all the acid gels at pH 4.5, no matter the PSH value of the complexes or the value of the G'_{max}. As discussed above, it seems that the constancy of tanδ across samples reflected the importance of the number of bonds, rather than their nature(s), on the increase in G' (Roefs & Van Vliet, 1990) Tanδ at pH 4.5 is also relatively independent on the microstructure of the particles inside the gel, while it is likely to account for the mechanical resistance and whey retention capacity of the protein network (Lakemond & Van Vliet, 2008). It therefore seemed that increasing the surface hydrophobicity of the whey protein complexes affected the building of the acid milk gels through increasing the number of possible connections in the 3 dimensions of space, thus affecting also the microstructure. This will be further discussed below.

The confocal images taken at pH 4.5 in the set acid gels are shown in Figure 41A, B and C. The protein network appears in white, while the pores appear dark. Image analysis using morphological closing to estimate the characteristic size of the pores showed that gels involving complexes of high PSH values (> 8000) had a higher mean mode of 4.1 µm as compared with all the other samples (1.5 ± 0.3 µm; $P > 0.5$, not shown). The ACP analysis confirmed that the scores of the various samples on the first component PC1 evidenced a reduction in the frequency of small pores (at 2 µm) and an increase in the frequency of large pores (at 7 µm) as the PSH values of the complexes increased (Figure 41D and E). These results indicated that larger pores were formed in gels that involved complexes with high surface hydrophobicity values, which probably accounted for their increased propensity to whey expulsion. These gels are also coarser, with more clusterized particles and less thin chains of particles, as compared to gels with complexes of medium PSH (Figure 41B and C). Unfortunately, we were not able to analyse the distribution of protein chains in the micrographs, as unexpected fields of high fluorescence of size 5-15 µm were visible in the gels

containing WPIA-C4$^-$ complexes (Figure 41A). This probably resulted from the decantation of insoluble material.

Interestingly, the present results bear some resemblance to those obtained when pre-renneting unheated or heated skim milk prior to acid gelation. The rennetting creates hydrophobic sites on the surface of the casein micelles, as the binding of the modified heat-induced complexes on the micelles is supposed to do. In agreement with our findings, studies also reported the significant increase in gelation pH, final gel strength and porosity of the resulting acid gels (Gastaldi et al., 2003; Li & Dalgleish, 2006; Lucey et al., 2000; Lucey et al., 2001; Niki et al., 2003).

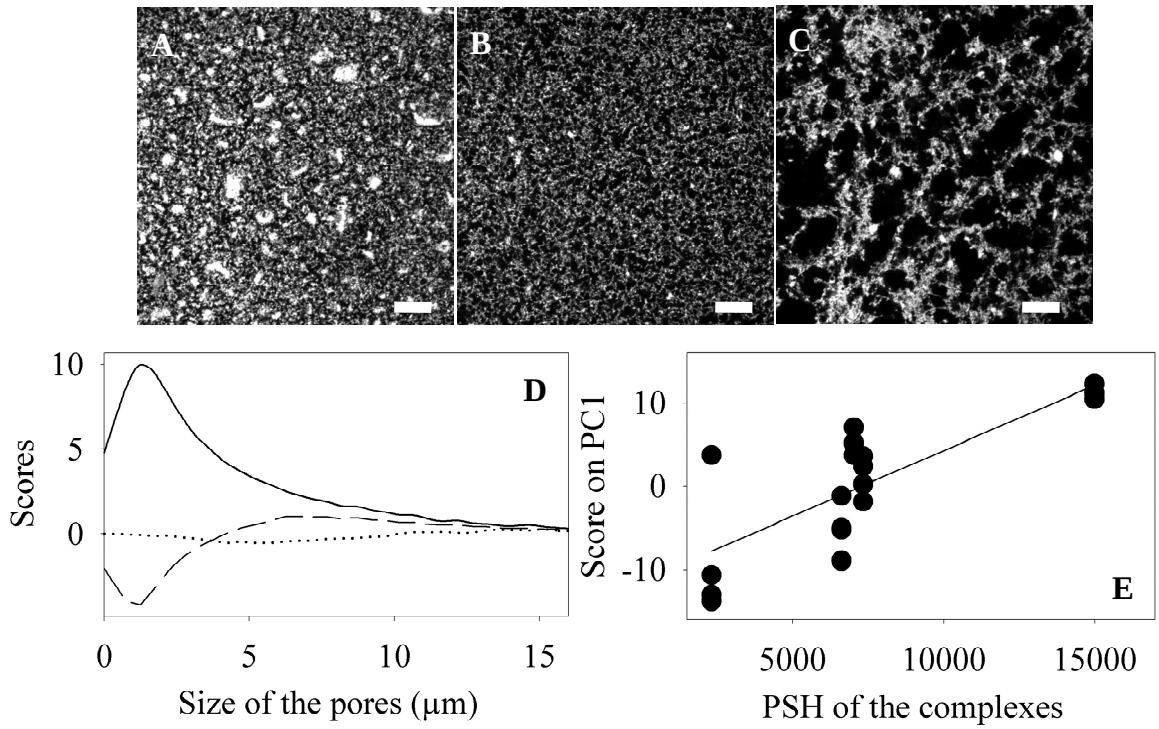

Figure 41 : Microstructure of the gels obtained from model milk systems at pH 4.5 observed by confocal laser scanning microscopy with complexes of increasing hydrophobicity (PSH): gel with complexes of (A) low PSH, (B) medium PSH and (C) high PSH values. Scale bar is 15 µm. (D) Mean size distribution of the pore sizes in the gels (—), PC1 (– – –) and PC2 (·····) as analysed by granulometric method. (E) Scores of the sample gels on PC1 as a function of the PSH of the complexes

Final gels at pH 4.5 were eventually characterised using large deformation rheology and penetrometry. The initial slope of the penetration force in the gel indicates the stiffness of the gels. The stiffness of gels involving complexes having PSH values of ~6500 (2.0 ± 0.7 N mm^{-1}) was higher than that of the other gels (1.0 ± 0.3 N mm^{-1}; $P < 0.01$). That result confirmed the

high G' value of the acid gels of the reconstituted milk samples with medium PSH complexes. The yield stress point indicates the resistance of the gel to fracture. The gels presented a mean resistance to fracture of 57 ± 31 Pa, without any difference between the various complexes, due to the low sensitivity of the method at that order of magnitude. In the light of the discussions by Roefs & van Vliet (1990a) or van Vliet et al. (1991), the fact that all the gels resisted in a similar manner to large deformation while having different G' values at pH 4.6 indicated that the consequences of modifying the number of hydrophobic sites on the whey protein complexes must be considered at various length scales. Possibly, gels with larger pores resulted from either the pre-aggregation of complexes or complexes/micelles into less numerous and larger adhesive particles, in the course of acidification, or from the local fusion of close protein strands inside the formed network, which would increase the mesh size, i.e. pore size, as fusion proceeds. Either way, the thickness and strength of the strands would increase and result in higher G' values. Macroscopically, the energy request for rupture at large deformation may however remain the same because all the gels are connected 3D-networks built with the same amount of the same type of material (similar bonds), only that the bonds are evenly distributed in space or not. To gain insight on the exact sequence of gel formation and rearrangement, kinetic studies should be envisaged using e.g. turbidimetry or confocal microscopy.

3.4. Balance of electrostatic repulsion and hydrophobic attraction on the pH of acid gelation

In order to cross the effects of the electrostatic and hydrophobic interactions on the pH of gelation of the suspensions of complexes and of the milk systems, all the samples shown in Figure 36, with pI values ranging 3.5-4.4, are now considered. In order to further increase the degree of freedom of the analysis, we also decided to consider preliminary data obtained during acid gelations at 35°C, even though the pH of gelation of acidified skim milk is known to somewhat depend on the temperature (Horne, 2001). The values of pH of gelation of all these systems were reported on Figure 42 as a function of the PSH of the complexes. In this figure, four groups of complexes are identified according to their pI (< 3.5; 3.5 to 3.9; 3.9 to 4.0; > 4.4).

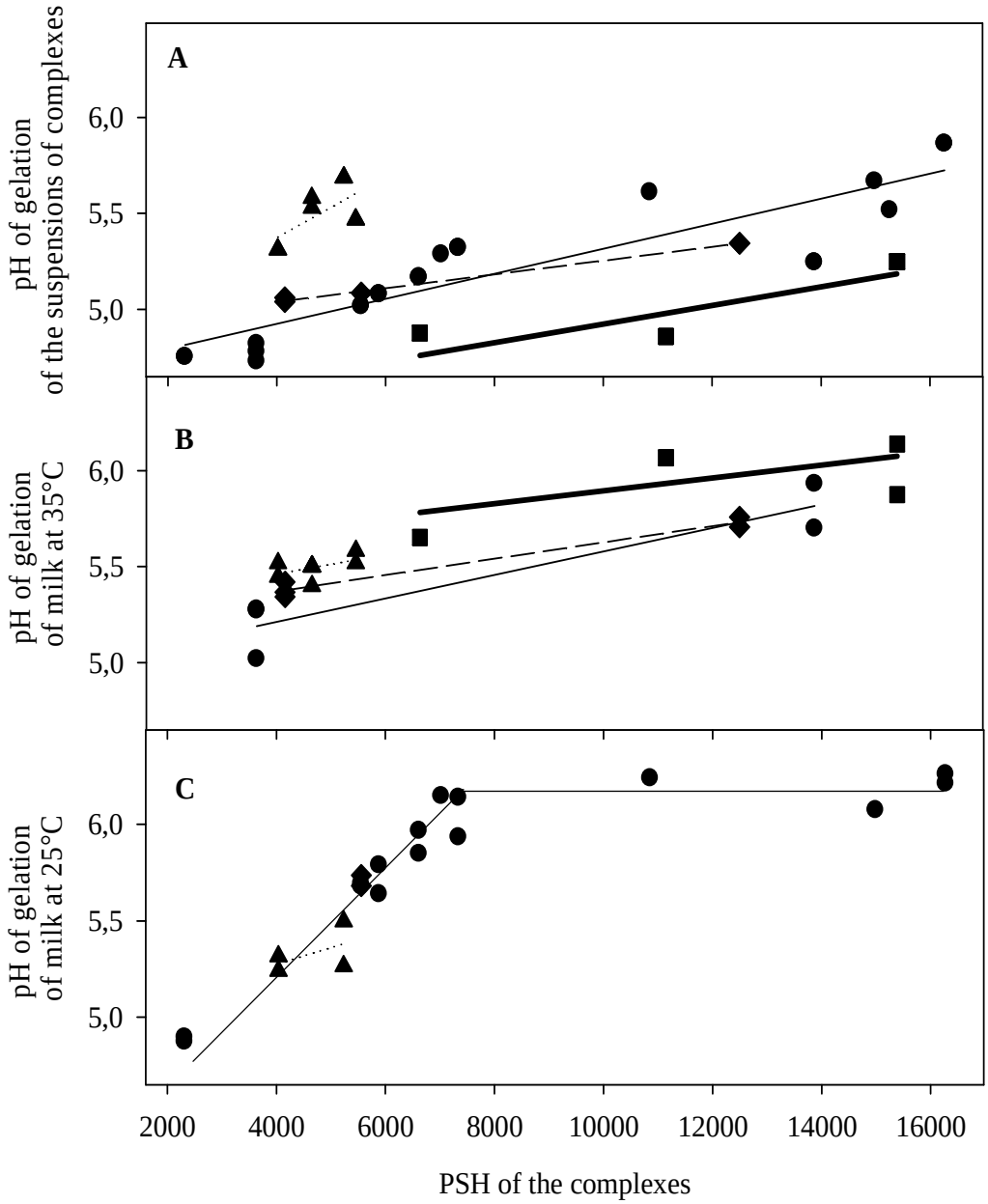

Figure 42 : pH of gelation of acid gels made from heat-induced complexes at 35°C (A), or from model milk systems containing heat-induced complexes at 35°C (B) and at 25°C (C) as a function of the surface hydrophobicity of complexes (PSH) and of their isoelectric point (pI): pI < 3.5 (—■—), 3.5 < pI < 3.9 (—●—), 3.9 < pI < 4.4 (– – ◆ – –) and pI > 4.4 (┈┈▲┈┈). The curves are for the linear regressions for each class of pI

Figure 42A evidences that the pH of gelation of the complexes alone depended both on the PSH and on the pI of the heat-induced complexes. For each pI-group of complexes, the pH of gelation increased as the PSH was increased, and when considering all the 4 groups, the pH of gelation increased as the pI was increased (Morand et al., 2012). However, the slopes of the 4 regressions were quite similar to one another, except for the group with high pI (pI > 4.4) which showed a higher dependence of the pH of gelation on PSH than the other groups. But

the PSH range of this group was very small (4000 < PSH < 6000). This evidences that an interaction may exist between electrostatic repulsion and hydrophobic attraction in driving the destabilisation of the whey complexes alone, as the increase in PSH at a low or high pI had quantitatively not the same effect on the gelation pH.

Figure 42B and C show that the pH of gelation of milk systems either at 35°C or 25°C also increased as the PSH value of the added complexes was increased. However this time, the slopes of the regressions were the same across the four pI-groups, i.e, no interaction could be observed between electrostatic repulsion and hydrophobic attraction. Unfortunately, we did not have enough data points to accurately evaluate the interaction between electrostatic repulsion and hydrophobic attractions.

According to Morand et al. (2012), changing a property of the complexes generates slightly lower shifts of the gelation pH in milk systems than in the suspensions of complexes. This is probably due to the 80% w/w of casein proteins in the milk systems that obviously take part in the gelation process. This result confirmed that despite the lower proportion of the heat-induced complexes relative to the casein micelles, they have a highly significant role in determining the stability of the milk systems.

In the literature, the stability of casein micelle colloids is usually discussed using the theory developed by Deryagin, Landau, Verwey and Overbeek, so-called the DLVO theory, which calculate the resultant free energy between attractive van der Waals and repulsive electrostatic interactions, as a function of the distance away from the particle surface. In the case of casein micelles, steric repulsion by the negatively charged and hydrated κ-casein brush is also reported to significantly contribute to colloidal stability at pH ≥ 6.0 (de Kruif, 1999; de Kruif, 1997; Tuinier & de Kruif, 2002; Walstra, 1990). During acidification, ionisation of the charged groups on the protein chains decreases, which reduces the repulsive steric and electrostatic interactions to the extent that aggregation can take place. This model accounts very well for the pH of gelation of milk (Tuinier & de Kruif, 2002). However, the possible contribution of hydrophobic interactions to the attractive forces or to affecting the energy barrier is not considered, even when studying the effect of prerenneting milk on the acid stability of casein micelles, probably because the actual origin of hydrophobic force is still unknown. Models to describe hydrophobic interaction usually refer to changes in the organisation of the water

molecules in the vicinity of the hydrophobic sites on two facing particles, leading to either local water depletion or to dipole-dipole hydrogen bonds mediated by the water molecules (Israelachvili, 2011). In that respect, the link with DLVO theory could be through the solvent quality parameter that strongly affects the free energy profiles (Walstra, 2003b). In the present study, a constant solvent phase, e.g. the MUF, may be a good solvent for the un-modified particles (whey protein complexes or micelles coated with complexes) but a poor solvent when the surface hydrophobicity of the same particles has been increased through chemical modification. This would then account for the higher pH of gelation of the casein micelles when in presence of complexes with a high surface hydrophobicity.

4. Conclusions

The results clearly showed that increasing the surface hydrophobicity of the whey protein heat-induced complexes increased the pH of gelation and the gel strength of acid gels of complexes or of model milk, although the effect on gel strength was counteracted by the increasing porosity and whey expulsion of gels having complexes of excessive surface hydrophobicity. A balance between attractive and repulsive forces determines the stability of milk particles and the gelation point. We have changed this balance by tuning both the pI and the PSH values of the complexes. Unfortunately, we did not have enough data points to accurately evaluate the interaction between electrostatic repulsion and hydrophobic attractions. Further research is also needed to evaluate the contribution of other forces on determining the pH of gelation of acidified skim milk, like van der Waals interaction, hydration and hydrogen bonds and steric repulsion, with respect to electrostatic repulsion and hydrophobic attraction.

Acknowledgements

This work was financially supported by Région Bretagne, under the grant ARED 4298.

VI.3. Bilan de la partie 3

Dans cette partie de résultats, nous avons largement modifié l'hydrophobie de surface (PSH) des complexes thermo-induits (entre ~2000 et ~16000) sans que les autres propriétés physico-chimiques ne soient modifiées. L'augmentation du PSH des complexes entraîne l'augmentation du pH de gélification des systèmes laitiers (suspensions de complexes et laits reconstitués avec les complexes, Figure 43). De plus, augmenter modérément l'hydrophobie des complexes (PSH ~5000-8000) permet une augmentation significative de l'élasticité des gels acides (G'_{max}, Figure 43). Toutefois, une hydrophobie des complexes trop importante (PSH > 10000) aboutit à la fragilisation des gels acides et l'expulsion de sérum.

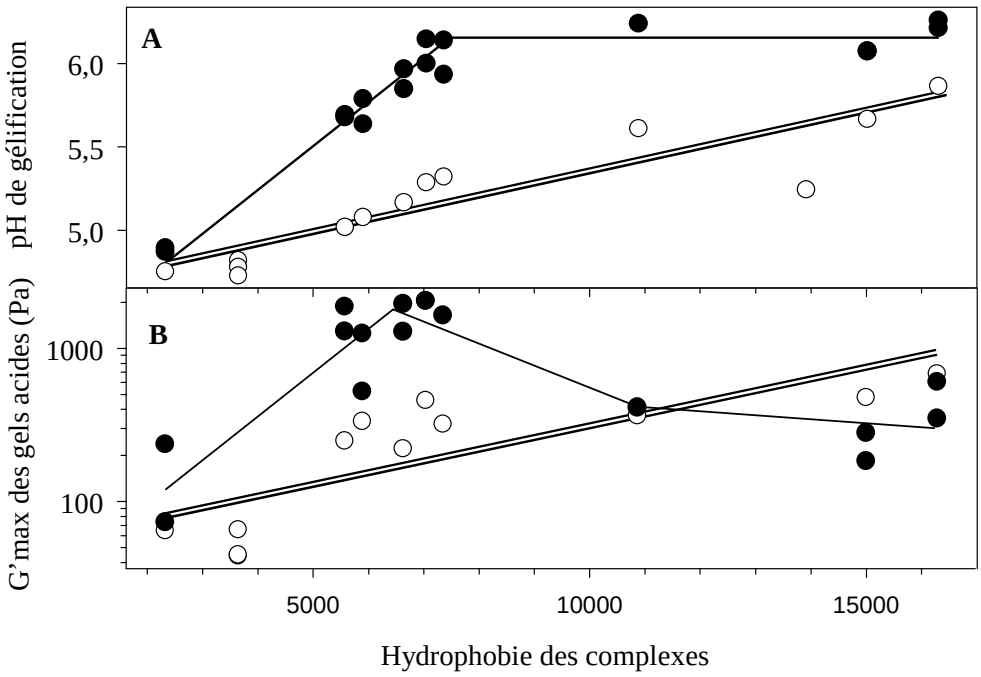

Figure 43 : (A) pH de gélification des systèmes laitiers (suspensions de complexes (○) à 35°C ou laits reconstitués avec les complexes (●) à 25°C) et (B) G'_{max} des gels acides en fonction de l'hydrophobie des complexes thermo-induits

Des complexes plus hydrophobes permettraient le renforcement de la structure des gels acides par l'augmentation du nombre d'interactions hydrophobes. Nous avons mis en évidence une interaction (faible) entre les répulsions électrostatiques et les attractions hydrophobes dans la déstabilisation des suspensions de complexes. Par contre, cette interaction n'est pas visible dans le cas des laits.

Chapitre VII.

Conclusion générale

Nous avons réussi à moduler, à façon, les propriétés de charge et d'hydrophobie de surface des complexes thermo-induits sans que les autres propriétés physico-chimiques (taille, structure secondaire et groupements thiols) ne soient significativement altérées. Ces deux propriétés se sont avérées fortement influentes sur la déstabilisation des systèmes laitiers (pH de gélification des suspensions de complexes et des laits reconstitués avec les complexes) et sur les propriétés des gels acides, dont une représentation schématique est proposée sur la Figure 44. L'augmentation du point isoélectrique des complexes ou l'augmentation de l'hydrophobie des complexes ont permis d'obtenir des pH de gélification plus élevés. L'augmentation de l'hydrophobie des complexes thermo-induits semble un bon moyen pour augmenter le pH de gélification des systèmes laitiers tout en augmentant la force des liaisons constitutives des gels acides laitiers. Toutefois, nous avons observé l'expulsion du sérum dans les gels acides de laits reconstitués avec des complexes très hydrophobes, ce qui ne nous a pas permis de mesurer les G'. Néanmoins, ces gels étaient contractés et élastiques, nous supposons donc des forces de liaisons plus importantes dans ces gels acides.

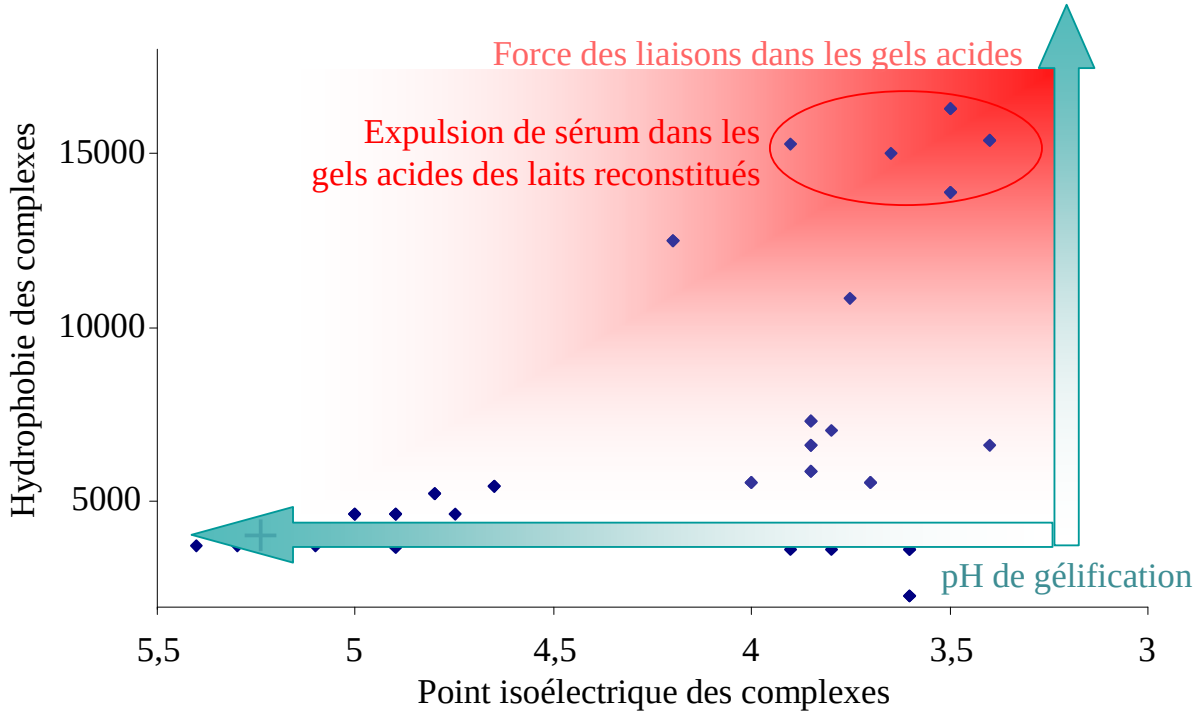

Figure 44 : Schéma général illustrant l'influence du point isoélectrique et de l'hydrophobie des complexes thermo-induits sur les propriétés de gélification des systèmes laitiers

Nous n'avons pas exploré la zone correspondant à des complexes très hydrophobes avec un pI élevé. Pour cela, il faudrait, par exemple, réaliser des greffages de chaines carbonées sur des groupements non chargés des complexes. D'autres alternatives sont envisageables : des réactions d'estérification de molécules d'alcool sur les complexes, la succession des réactions d'acylation et de méthylation des complexes… La fixation du rouge de Nile sur les complexes, envisagée dans la revue bibliographique (§II.6.2.2) pourrait constituer un autre moyen d'augmenter l'hydrophobie des complexes. Elle ne s'établirait pas de façon covalente mais nécessiterait de vérifier sa stabilité après le mélange avec les micelles de caséines ou encore pendant l'acidification. En effet, le rouge de Nile se fixe sur les zones hydrophobes des protéines et pourrait donc se décrocher des complexes pour aller se fixer sur les micelles de caséines. Il reste également à tester des moyens compatibles avec l'alimentation pour modifier le pI et/ou l'hydrophobie des complexes, comme par exemple l'ajout de protéines non laitières ou la Maillardisation qui apporteraient de nouvelles propriétés aux complexes, par fixation de sucres réducteurs neutres ou chargés.

Nous avons observé que l'effet des modifications des complexes testées est plus marqué sur les propriétés de gélification des suspensions de complexes que sur celles des laits reconstitués. Les effets observés sur les suspensions complexes reflètent des interactions complexes-complexes au cours de l'acidification tandis que ceux sur les laits reconstitués reflètent des interactions complexes-complexes et complexes-micelles. Les effets reflètent aussi de la plus grande complexité du lait par rapport aux suspensions de complexes seuls, notemment si on considère que la micelle de caséines n'a pas une organisation macrocopique constante au cours de l'acidification. Quoi qu'il en soit, les tendances observées sont les mêmes dans les deux systèmes et il apparaît que les complexes ont imposé leurs propriétés à la micelle de caséines malgré leur faible teneur dans les laits reconstitués (20% w/w des protéines). Cette avancée va dans le sens de l'hypothèse de la fonctionnalisation de la surface de la micelle par les complexes thermo-induits au cours du traitement thermique d'une part (complexes micellaires) et au cours de l'acidification et ce avant la gélification (complexes solubles).

Les résultats de cette étude participent à la compréhension des mécanismes de déstabilisation du lait et de la construction des gels acides. Nous nous sommes limités aux interactions électrostatiques et hydrophobes mais d'après la littérature, d'autres facteurs, en particulier, les échanges thiols/disulfures, les liaisons hydrogènes et les gênes stériques (taille et forme des complexes), devront être étudiés pour compléter l'actuel mécanisme de gélification du lait proposé ci-dessous :

1. Fonctionnalisation de la micelle de caséines par les complexes thermo-induits

   Au cours du traitement thermique ou au début de l'acidification, les complexes et la micelle de caséines interagissent et les complexes « tapissent » la surface de la micelle. Les complexes fonctionnalisent la micelle, lui apportant leurs propriétés physico-chimiques.

2. Gélification

   La réduction des répulsions électrostatiques par l'acidification permet le rapprochement des particules et l'établissement d'interactions hydrophobes qui jouent un rôle significatif, ainsi que d'autres intéractions qui restent à élucider.

3. Réarrangements des liaisons du gel acide

   Au cours de l'acidification et après la gélification, les particules peuvent établir / rompre des interactions avec leurs voisines. Lorsque la force des liaisons est trop forte, les réarrangements induisent la contraction locale du gel et l'ouverture de pores, ce qui peut conduire à la fragilisation du gel et à l'expulsion de sérum.

4. Fermeté à pH 4.5

   Le gel final acide final résulte de l'ensemble des interactions intra et interparticulaires.

L'hypothèse de la fonctionnalisation de la surface de la micelle par les complexes thermo-induits au début de l'acidification reste encore malgré tout à être vérifiée. En particulier, suite à la modification des propriétés de surface des complexes, leurs interactions avec la micelle peuvent être modifiées. Une approche similaire à celle utilisée par Guyomarc'h et al. (2009) pourrait être envisagée. Elle consiste à marquer séparément la micelle de caséines et les complexes thermo-induits par des sondes fluorescentes de spectres d'excitation différents et de suivre la localisation des sondes dans le réseau protéique au cours de la gélification en microscopie confocale. Cette approche permettrait également de vérifier l'interaction complexes-micelles par la méthode de transfert d'énergie de résonnance (méthode de FRET) de 2 sondes distantes de 1 à 10 nm.

En perspective, nous pourrons bientôt enrichir ce mécanisme par les résultats d'une thèse actuellement en cours dans le groupe Fonterra en Nouvelle-Zélande portée par Nguyen Nguyen et co-dirigée par Skelte Anema et Fanny Guyomarc'h sur le rôle de la composition en thiols des complexes thermo-induits sur les propriétés de gélification acide du lait. Un nouveau projet de thèse débutera en novembre 2011 à l'UMR STLO avec un étudiant Indonésien, Robi Andoyo, sous la direction de Marie-Hélène Famelart et Fanny Guyomarc'h, qui poursuivra cette étude. Ce nouveau projet abordera également le rôle de la taille et la forme des complexes thermo-induits et les autres propriétés des complexes comme l'aptitude à former des ponts hydrogènes sur les propriétés de gélification acide du lait.

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