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STUDY OF ENZYMATIC PREPARATION OF CELL WALL RESIDUE

ETUDE DES CONDITIONS DE PRÉPARATION DE RÉSIDUS PARIÉTAUX PAR VOIE ENZYMATIQUE

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SUMMARY

Study of *in vitro* fermentation of non starchy polysaccharides involves the preparation of cell wall residues by protein and starch enzymatic hydrolysis. Protein and starch removal by chemical or enzymatic treatment is used in the analytical methods to determine dietary fibre content in feed or food products (SELVENDRAN *et al.*, 1975; PROSKY *et al.*, 1985; BRILLOUET *et al.*, 1988) ; but the procedures are inaccurate as regards obtaining large amount of cell wall residue. In this work, the preparation of cell wall residues was studied by enzymatic digestion using commercial mixtures (Alcalase 0.6 L, Termamyl 120 L, AMG 200 L, Novo).

Plant substrates (wheat bran, beet pulp, corn germ meal, pea fibre) were ground and defatted by the mixture chloroform/methanol. For optimizing the protein digestion stage, Alcalase activity and proteolysis parameters were studied: pH (6.0, 7.5), temperature (50°C, 60°C) and rate enzyme/substrate. Under maximal proteolysis conditions (60°C, pH 7.5, 2 hours), protein solubilisation was 27%, 46%, 45% and 55%, respectively for wheat bran, beet pulp, pea fibre and corn germ meal; increasing losses of arabinose and galacturonic acid occured after 2 and 24 hours in different conditions of incubation. Same proteolysis efficiency was obtained with a lower temperature (50°C), therefore the selected proteolysis conditions were: incubation with Alcalase for 2 hours at 50°C and pH 7.5. Starch was completely removed by action of a heat-stable a-amylase (Termamyl 120 L) (100°C during 30 min) and an amyloglucosidase (AMG 200 L).

Key-words: *dietary fibre, plant cell wall, enzymatic hydrolysis.*

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RÉSUMÉ

La préparation de résidus pariétaux est nécessaire pour l'étude *In vitro* des fermentations des polyosides non amylacés. Pour déterminer la teneur en fibres alimentaires des aliments, l'élimination des constituants cytoplasmiques (protéines, amidon) s'effectue par des méthodes chimiques ou enzymatiques (SELVENDRAN *et al.*, 1975 ; PROSKY *et al.*, 1985 ; BRILLOUET *et al.*, 1988). Ces méthodes analytiques sont inadaptées à la préparation de fractions pariétales. L'objet de ce travail est d'étudier les conditions de préparation de fractions "fibre alimentaire" par traitements avec des mélanges enzymatiques commerciaux (Alcalase 0,6 L, Termamyl 120 L, AMG 200 L, Novo).

Les matières premières (son de blé, pulpe de betterave, tourteau de germes de maïs, fibres de pois) sont broyées et délipidées par un mélange chloroforme/méthanol. L'activité protéasique de l'alcalase 0,6 L et différentes conditions de protéolyse (pH : 6,0 et 7,5 ; température : 50 et 60°C, rapport enzyme/substrat) ont été étudiées. Dans les conditions optimales de protéolyse (60°C, pH 7,5 , 2 heures) la solubilisation des protéines pour les substrats étudiés est respectivement de 27 %, 46 %, 45 % et 55 % pour le son de blé, les pulpes de betterave, le tourteau de germes de maïs et les fibres de pois. La même efficacité de protéolyse est obtenue pour le tourteau de germes de maïs, lorsque la température de protéolyse est de 50°C ; de plus, des pertes croissantes d'arabinose et d'acide galacturonique sont constatées après 2 et 24 heures d'incubation à pH 6,0, 50°C et pH 7,5 , 60°C. Les conditions optimales de protéolyse ainsi déterminées (pH 7,5 , 50°C, 2 heures) seront donc conservées. La digestion de l'amidon s'effectue par l'action successive d'une α - amylase thermostable (Termamyl 120 L) à 100°C pendant 30 min, et d'une amyloglucosidase (AMG 200 L).

Mots clés : *fibre alimentaire, paroi végétale, hydrolyse enzymatique.*

1 - INTRODUCTION

Dietary fibres include complex non starchy plant polysaccharides (cellulose, hemicellulose, pectic substances, gums, mucilages, etc.) and lignin. They are not degraded by human gut enzymes, but they are fermented by colonic bacteria, resulting in the formation of gases (H_2, CH_4, CO_2) and volatile fatty acids, VFA (acetic acid, butyric acid, propionic acid, valeric acid) ; the proportion of VFA produced and the rate of dietary fibre degradation depend on chemical composition and physical properties of dietary fibre. *In vivo* studies on dietary fibre fermentation in human are difficult, also *in vitro* approach, performed with dietary fibre residue can be used. These experiments require large amounts of cell wall samples. Procedures of dietary fibre purification are actually provided for analytical purpose, i.e.: the gravimetric determination of total or insoluble dietary fibre residues (PROSKY *et al.*, 1985; BRILLOUET *et al.*, 1988). Purified enzymes are usually used in the enzymatic hydrolysis stage, mainly proteolytic enzymes. In this work we have developed a procedure using commercial

enzymes (food grade) to prepare dietary fibre residues suitable for the *in vitro* experiment. Proteolytic enzymes have usually optimal conditions of use (pH, temperature) which are unsuitable for preserving the chemical structure of cell wall polysaccharides (ASP *et al.*, 1983). In this work, we have studied proteolysis and amylolysis conditions that would not degrade cell wall polysaccharides.

2 - MATERIALS AND METHODS

2.1 Samples and samples preparation

Wheat bran (coarse) and beet pulp (dried and pelleted) were provided by Cana (Ancenis, France). Corn germ meal was purchassed from Roquette (Lille, France) and fibre pea was prepared at "Institut National de la Recherche Agronomique" (Nantes, France). Wheat bran and beet pulp were ground to pass through a 3 mm screen. All samples (200 g) were defatted by about 300 ml of chloroform/methanol mixture (2:1, v/v) during 15 hours, filtered on a sintered-glass crucible (porosity 4), dried by acetone, ethanol, ether and kept (overnight) in a vacuum oven at 40°C and weighed.

2.2 Enzymes and chemicals

All enzymes used for cell wall preparation were provided by Novo Industri A/S (Denmark): Alcalase 0.6 L (*Bacillus licheniformis*, 0.75 µkat/ml), Termamyl 120 L (E.C.3.2.1.1, from *Bacillus licheniformis*, 95 µkat/ml), Amyloglucosidase 200 L (E.C.3.2.1.3; AMG, from *Aspergillus niger*, 147 µkat/ml). Enzymatic activity was expressed in µkatals; 1 µkatal is defined as the amount of enzyme which release 1 µmole of reaction product per seconde. Protease activity of Alcalase 0.6 L was determined by mixing enzyme (~ $2x10^{-3}$ µkat) with solution of bovine serum albumin (2% in phosphate buffer 0.1 M, pH 7.5) at 40°C; after the incubation, mixture was precipitated with TCA 20%; tyrosine-equivalent was measured in the supernatant by the Folin method. Amylase activity was determined by incubating Termamyl (~ $0.4x10^{-3}$ µkatal) with soluble starch (Merck, 2% in phosphate buffer 0.1 M, pH 6.0, CaCl2 0.3 mM); the released reducing sugars were measured by the method of NELSON (1944), using maltose as standard. Activity of AMG 200 L was measured by following release of glucose from maltose by the enzymatic method, GOD/POD (THIVEND *et al.*, 1972); the assay mixture containing enzyme (~ $7x10^{-3}$ µkatal) and maltose (1% in citrate buffer (0.1 M, pH 4.5) was incubated at 60°C.

Enzymes were checked for contaminating activities on the following substrates: carboxymethyl cellulose sodium salt (Sigma), polygalacturonic acid (I.C.N.), oat spelt xylan (Sigma), arabinogalactan (Serva), oat glucan (Rapidase). Substrates (1%) were incubated with dialysed enzymes (Alcalase 0.6 L: 0.023 µkatal; AMG 200L: 0.4 µkatal) in standard conditions of activity

measurement for each enzyme and liberated reducing sugars were determined by the NELSON procedure (1944), using glucose as a standard.

2.3 Analytical methods

Moisture content was determined by drying overnight at 100°C. Ashes were measured by incinerating at 550°C for 5 hours. Nitrogen was measured by the Kjeldahl method and converted in crude protein using correction factor 6.25. Starch (100 mg) was determined by dispersion in DMSO 98% (25 ml) at 110°C for 1 hour. After cooling, solution was adjusted to 100 ml with water. Aliquot was hydrolysed by amyloglucosidase (Merck, 100 UI) overnight at 30°C, in acetate buffer pH 4.8; glucose released was determined according to Bœringher UV method (ref. 716 251). Starch was also detected by iodine test.

Cell wall neutral sugars, released by acid hydrolysis, were quantified by gas-liquid chromatography as their alditol acetates according to the simplified procedure of HOEBLER *et al.* (1989). Inositol was used as internal standard.

Uronic acids released by acid hydrolysis were analysed by m-phenylphenol (MHDP) method (THIBAULT, 1979).

2.4 Proteins and starch digestion

For studying proteins digestion, ground and defatted samples (1 g) were weighed in conical flask; they were thoroughly mixed with phosphate buffer 0.1 M, pH 6.0 or 7.5 (25 ml) and put in a water bath (50°C or 60°C); Alcalase 0.6 L (0.375 µkat) was added, and the mixture was incubated during 0, 0.25, 0.5, 1, 2 and 20 hours. Mixtures were precipitated by adding four volumes of ethanol 98% and left 60 minutes at room temperature. Mixture was filtered on sintered glass crucible, washed by acetone, ethanol, ether; the residue was dried overnight at 40°C and weighed. For starch digestion, substrates were incubated with appropriate amount of Termamyl in phosphate buffer 0.1 M, pH 6,0 and remained for 0.5 h in boiling water bath. After cooling, pH was brought to 4.5 with 1.5 ml HCl 2 N. Amyloglucosidase (44,1 µkat) was added and the flask was kept for 0.5 h at 60°C.

3 - RESULTS AND DISCUSSION

Substrates composition are indicated in table 1. All enzymes used in this study were commercial mixtures and were tested for contaminating activities on various polysaccharides (see Materials and methods). Alcalase 0.6 L exhibited very low polygalacturonase, xylanase and glucanase activities (below 0.01% of proteolytic activity); the amyloglucosidase (AMG 120 L) was free of contaminants. It has been already reported that Termamyl 60 L had low foreign activity ($< 2 \times 10^{-3}$ % of amylase activity; BRILLOUET *et al.*, 1988).

Table 1
Non cell wall contents of crude materials

Materials	Dry matter (%)	Ash (% DM) (*)	Defatted extract (% DM) (*)	Protein N x 6.25 (% DM) (*)	Starch (% DM) (*)
Wheat bran	86.6	5.4	6.3	14.2	11.7
Corn germ meal	94.6	1.0	8.0	24.6	9.7
Beet pulp	89.7	7.2	0.9	7.6	0.5
Pea fibre	84.6	2.5	0.0	2.0	11.0

(*) Dry matter.

3.1 Protein digestion

A bacterial proteolytic enzyme (Alcalase 0.6 L) was used to hydrolyse cytoplasmic proteins. In optimal conditions of Alcalase 0.6 L utilisation (pH 8.5, temperature 60°C) cell wall polysaccharides can be degraded, mainly pectic substances by ß elimination (ALBERSHEIM *et al.*, 1960); for this reason, optimal temperature activity and thermal stability have been studied at pH 6.0 and 7.5.

Alcalase 0.6 L exhibited maximum activity at 60°C determined at pH 6.0 and 7.5 *(fig. 1A)*; its residual activity was determined after 0, 1, 2, 4 hours, in various conditions of incubation (pH 6.0, 7.5; temperature 50°C, 60°C) *(fig. 1B)*. Alcalase activity was quickly reduced at 60°C, it is tightly modified at 50°C *(fig. 1B)*. After 1 hour of incubation at pH 7.5 and 60°C, only 20% of maximal activity was recovered. Residual activity measured at pH 6.0 and 50°C was very low (16% of maximal activity). Rate of protein degradation (pea protein) was tested with increasing ratio enzyme/substrate (volume/weight, ml/g of substrate) *(fig. 1C)*.

The same ratio, Alcalase 0.6 L (0.375 µlat)/g substrate was selected for following assays to test farthest working parameters of Alcalase 0.6 L (pH 7.5, temperature 60°C; pH 6.0, temperature 50°C). Nitrogen solubilisation was higher and faster at pH 7.5 (temperature 60°C) than at pH 6.0 (temperature 50°C), mainly for corn germ meal *(fig. 2A and 2B)*; for this last substrate, same proteolysis curve was obtained at pH 7.5 at 50°C and 60°C; pH seemed to be important parameter for protein solubilisation. Proteolysis levels of wheat bran and pea fibre were similar in both conditions *(fig. 2A and 2B)*.

Under maximal proteolysis conditions (pH 7.5, 60°C), residual proteins were 8.1%, 3.5%, 1.6%, 8.2% (expressed in dry matter of residue) for wheat bran, beet pulp, pea fibre and corn germ meal respectively. When Alcalase was added after 24 hours of wheat bran incubation, residual nitrogen was not reduced, showing that all available proteins were solubilised.

No loss of neutral and acid sugars from beet pulp were observed after 1 hour of incubation with Alcalase under the different tested proteolysis conditions. After 2 hours of proteolysis at 60°C, pH 7.5, 90% of arabinose, 94% of galactose and 99% of galacturonic acid were recovered. When the proteolysis duration reaches 20 hours sugars losses increased (12% of arabinose and 10%

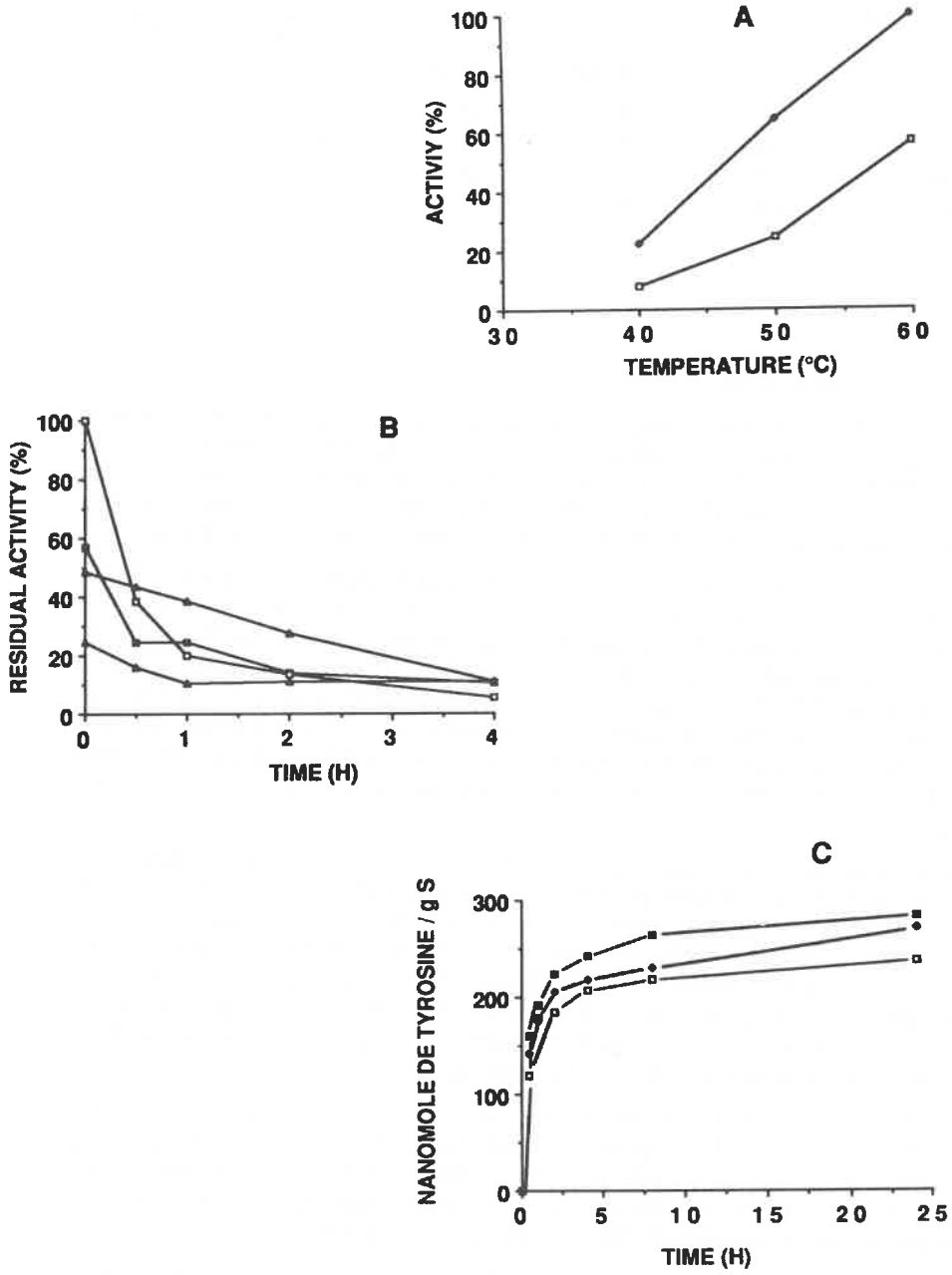

Figure 1
Working paramaters of Alcalase 0.6 L

(A) Effect of temperature on Alcalase activity at pH 6.0 (□), and pH 7.5 (●)
(B) Heating stability of Alcalase, (▲) pH 6.0, T°: 50°C ; (■) pH 6.0, T° : 60°C ; (△) pH 7.5, T° : 50°C; (□) pH 7.5, T°: 60°C
(C) Influence of the Alcalase 0.6 L (μkat) to the pea protein (g) ratio on the proteolysis rate: (□) 0.015/g, (●) 0.375/g, (■) 0.750/g performed at pH 7.5, 60°C.

of galacturonic acid, when proteolysis was carried out at pH 6.0, 50°C and 16% of arabinose and galacturonic acid, when proteolysis was carried out at pH 7.5, 60°C). These results indicated that the pectic substances were only a little degraded after 2 hours of proteolysis and they could be recovered by ethanol 80% precipitation.

According to these results, the conditions selected for proteolysis step to obtain maximal proteins solubilisation without loss of cell wall sugars were: pH 7.5, temperature 50°C, 2 hours of incubation. Residual proteins in cell wall residues were higher than protein level found by BRILLOUET *et al.*, 1988. However, the aim of this work was to optimize proteolysis conditions with substrates of which the selected particle sizes were closed to those found in physiological conditions.

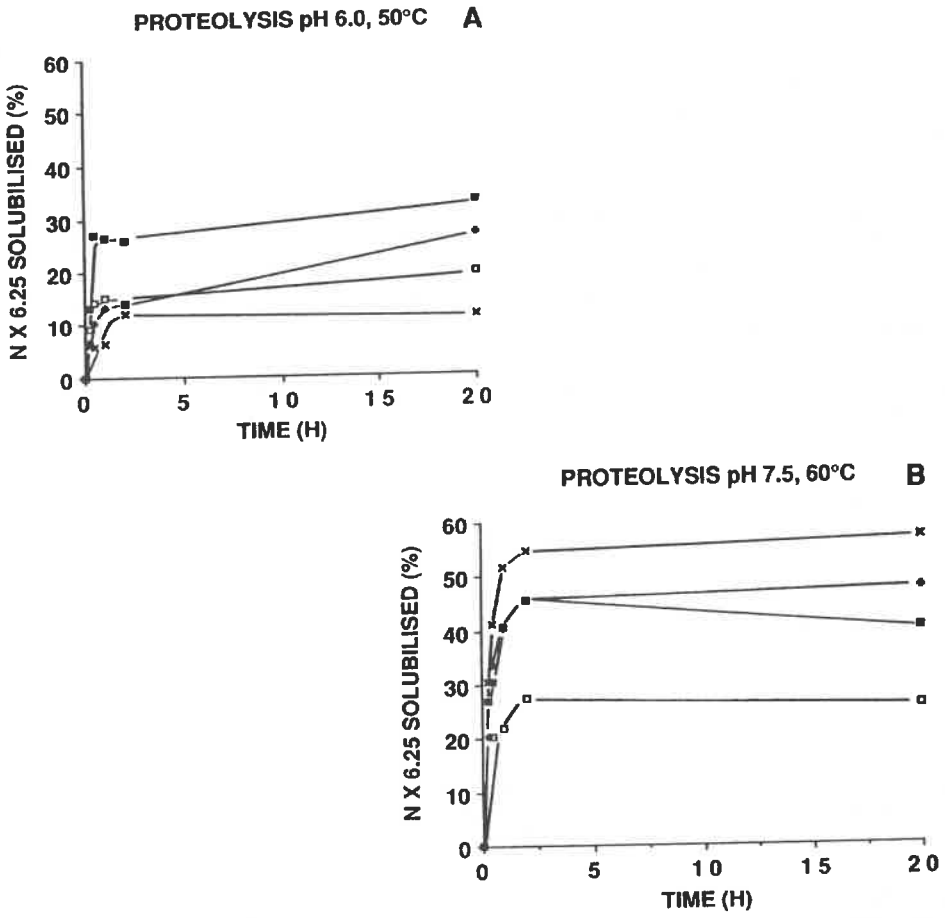

Figure 2
Enzymatic hydrolysis of proteins from (□) wheat bran, (x) corn germ meal, (♦) beet pulp, (■) pea fibre by Alcalase 0.6 L at pH 6.0 and 7.5

3.2 Starch digestion

Heat stable α-amylase, Termamyl 120 L and amyloglucosidase 200 L were used for the starch solubilisation and saccharification. Various amounts of Termamyl 120 L were added, (19, 47.5, 98 µkat/g of wheat bran); iodine reaction was tested on samples; it was negative in all the assays. The following amylolysis conditions were selected to ensure a complete removal of starch whatever the substrates: incubation of 0.5 ml Termamyl (47.5 µkatal) at pH 6.0 for 30 min and 0.3 ml AMG (44.1 µkatal) at pH 4.5 for 30 min.

4 - CONCLUSIONS

In this work, the residues obtained after proteolysis, were contaminated by residual nitrogen, but the samples recovered after the amylolysis step were free of starchy contamination.

According to the results of this study, the whole proposed procedure to prepare cell wall residue would include a proteolysis step, performed with Alcalase 0.6 L under determined conditions, followed by starch removal with Termamyl and amyloglucosidase incubation. After these enzymatic treatments, soluble dietary fibre would have been precipitated by ethanol 80%; soluble and insoluble fractions would be recovered by filtration through a glass crucible and dried. In further study, cell wall residue would be prepared according to the described method and chemical composition of samples obtained *in vitro* and *in vivo* (at the end of small intestine in pig) will be compared.

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