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Non-linear properties and yielding of enzymatic milk gels

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One of the first steps of cheese making is to suppress the colloidal stability of casein micelles by enzymatic hydrolysis and initiate milk gelation. Afterwards, the enzymatic milk gel is cut to promote syneresis and expulsion of the soluble phase of milk. Many studies have reported on the rheological properties of enzymatic milk gels at small strain, but they provide limited information on the ability of the gel to be cut and handled. In this study, we aim to characterize the non-linear properties and the yielding behavior of enzymatic milk gels during creep, fatigue and stress sweep tests. We evidence by both continuous and oscillatory shear tests that enzymatic milk gel displays irreversible and brittle-like failure, as reported for acid caseinate gels, but with additional dissipation during fracture opening. Before yielding, acid caseinate gels display strain-hardening only, while enzymatic milk gels also display strain-softening. By varying the gel aging time and the volume fraction of casein micelles, we are able to attribute the hardening to the network structure and the softening to local interactions between casein micelles. Our study highlights the crucial importance of the nanoscale organization of the casein micelles – or more generally of the building block of a gel – to retain the macroscopic nonlinear mechanical properties of the gel.

1 Introduction

Many dairy products are consumed as gels or involve a gel state in their manufacturing process. The most known examples are (i) yogurt, formed by slow acidification of milk to pH $\approx$ 4.6 and (ii) cheese curd, formed by the addition of proteolytic enzymes at pH 6.6 or lower.[1] These gels are built of casein micelles (CMs) which are natural colloidal particles, resulting from the association of phosphorylated proteins, caseins, and milk minerals, mainly calcium and phosphate.[2] CMs are spherical and "soft" particles, having an apparent Young's modulus $E^* \approx 0.5$ MPa.[3] They display a size distribution centered at $\approx$150 nm in bovine milk. Being highly hydrated, these particles can be viewed as protein microgels that contain thousands of polypeptide chains and whose colloidal stability is ensured by a specific casein fraction protruding at the surface of the colloid.[4]

During cheese manufacture, milk gelation is induced by the addition of proteolytic enzymes that suppress the colloidal stability of CMs.[5] Destabilized CMs aggregate through weak interactions such as hydrophobic, van der Waals or electrostatic interactions. Consequently, enzymatic milk gels are weak physical gels,[6] with G' and G'' being of the same order of magnitude. Because of the intermediate volume fraction of CMs in milk $\phi_{\mathrm{CM}} \approx 0.1$ and the intermediate range of repulsive interactions between destabilized CMs,[7] enzymatic milk gels deviate from ideal fractal gels. As depicted for acid-induced sodium caseinate gels, the gel state results from an "arrested phase separation" between caseins and the soluble phase of milk.[8] This non-equilibrium state manifests itself by gel aging, leading to a coarse structure composed of glassy strands and large pores.[9,10] To be further transformed into cheeses, enzymatic milk gels are cut during aging, which promotes gel syneresis and expulsion of the soluble phase.

The rheological properties of enzymatic milk gels have been extensively studied in the linear domain,[11–15] especially to determine the optimal cutting time during processing. However, Van Vliet & Walstra[16] have pointed out that the ability of such gels to be cut and handled is much more related to their non-linear behavior. In the non-linear domain, the response of the material is no longer proportional to the imposed stress or strain, and the structure and/or properties of the material are modified during the test. If the applied stress and duration of the test are high enough, the material will yield and eventually fail. The "yielding" of a colloidal gel is the transition from a solid to a liquid state, involving macroscopic plasticity and/or microscopic damage and/or viscous flow, that precede failure.[8,16,17]

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Yielding and failure of a material depend on its average mechanical properties and on its "flaws", which are the nucleation spots for strain concentration and fracture growth. Failure is a multiscale phenomenon, going from molecular bond breaking to crack growth. By measuring the strain γ changes under application of different stress σ_0, Van Vliet *et al.*[14] reported that enzymatic milk gels display significant deformation before failure and that the strain at failure γ_f depended on the applied stress that sets the time scale of the experiment. They attributed this behavior to the linear viscoelastic properties of the gels. From the frequency f dependence of the loss tangent $\tan\delta$, they postulated that an important property of the bonds is that they are short-lived ($\tau < 1$ s) and a significant part of the stress σ_0 is dissipated by viscous flow before failure. Under application of constant stress[12] or a constant shear rate $\dot{\gamma}$,[18] it was also reported that the strain at failure decreased and that the gel became more brittle during aging. In contrast, increasing the measuring temperature increased the strain at failure.[12]

Away from enzymatic gelation, the non-linear properties of acid-induced sodium caseinate gels have been extensively characterized as a model for brittle soft colloidal gels.[8,19–21] In this system, caseins are assembled into small particles containing $\approx$15 polypeptide chains,[22] whose gelation is induced by a decrease of pH. During creep tests, a primary linear viscoelastic regime is followed by fracture nucleation and growth with little plasticity in between.[19] Moreover, the failure time τ_f was found to decrease as a power law of the applied stress, which is characteristic of materials displaying irreversible failure. Such characterization has not been attempted for enzymatic milk gels, preventing its classification among other materials.[8]

In recent years, the yielding of materials has also been studied using large amplitude oscillatory shear (LAOS) rheology, as the frequency of the test can be easily varied.[23] During stress or strain sweep tests, materials have been classified according to changes in the stress to strain ratios beyond the linear domain.[24] An increase or a decrease of the stress to strain ratio with the applied stress is called "strain-hardening" or "strain-softening", respectively. Although the elastic and viscous moduli G' and G'' provide information about the material response only at the excitation frequency, it is convenient to observe strain-softening and hardening by plotting G' and G'' as a function of stress or strain.[23] Unlike most soft gels, acid-induced caseinate gels display strain-hardening before failure.[8] To our knowledge, such tests have not been reported on enzymatic milk gels.

Besides creep and LAOS tests, the non-linear properties of acid-induced caseinate gels have been studied by measuring the stress relaxation after step strain.[21] Provided such strain-controlled tests, it is possible to go to amplitudes beyond the hardening regime and observe a reversible strain softening regime. In stress-controlled experiments this softening regime is unstable and leads to irreversible material failure.[8]

In this study, we aim to further characterize the non-linear and yielding properties of enzymatic milk gels, in light of the literature mentioned above. Thereby, aged enzymatic milk gels were subjected to creep, fatigue and stress sweep experiments.

2 Materials and methods

2.1 Milk sample preparation

Milk samples were prepared by dissolving low heat skimmed milk powder in ultrapure water to a final casein concentration of 29 g L^{-1}. Skimmed milk powder was prepared in house, as described elsewhere.[25] Sodium azide was added to milk as a biopreservative to a final concentration of 0.2 g L^{-1}. After 2.5 h of rehydration at 20 °C under stirring, the milk pH was adjusted to 6.60 ± 0.03 by dropwise addition of 1 M HCl. Then, the milk was left for equilibration at 20 °C for 12 h and was used within 5 days. Assuming a voluminosity for CMs of 3 mL g^{-1} of dry casein,[26] we calculated $\phi_{CM} \approx 0.09$ in reconstituted milk. To vary the volume fraction of CMs, the casein content of milk was concentrated $\approx$2 fold using a lab-scale tangential filtration system equipped with 10 kDa cut-off membranes (Centramate™ cassettes Medium screen OS010C12, Pall Corporation, Saint-Germain-en-laye, France). The milk ultrafiltration permeate was collected and used to dilute either milk or filtration retentate to prepare samples at $\phi_{CM} \in [0.05;0.19]$. Dilution using milk permeate rather than water ensures that no variation in the composition of the milk aqueous phase occurs, and consequently, the CM properties are maintained constant (*i.e.* the composition and ζ-potential). The total and non-casein nitrogen content of both milk and milk retentate were determined using the Kjeldahl method. The volume fraction of samples was then deduced from the casein concentration. Gelation was conducted at 30 °C. Milk aliquots were warmed 30 m at this temperature in a water bath, prior to coagulant addition. To initiate gelation, 20 μL of 1:100 diluted coagulant (CHY-MAX® SUPREME 1000 international milk clotting units mL^{-1}, Chr. Hansen, Hoersholm, Denmark) was added to 10 mL of milk.

2.2 Rheological measurements

All rheological measurements were performed using a stress-controlled rheometer (MCR 301, Anton Paar) equipped with a CC17 Couette geometry (cup diameter = 18.08 mm, bob diameter = 16.66 mm). The temperature was set at 30 °C using the thermoregulation system of the rheometer. After coagulant addition to milk, 4.5 mL of milk + coagulant were immediately loaded in the geometry and covered with paraffin oil to avoid evaporation. Gelation was monitored through small angle oscillatory shear at f = 0.1 Hz and γ = 1%. The gelation, approximated when $G' > G''$, occurred within 40 min after coagulant addition. The aging dynamics and the rheological properties in the linear domain of the enzymatic milk gels have been reported elsewhere.[27]

If not specified, rheological tests including creep, fatigue and stress sweep were initiated 260 min after the gelation time, when G' and G'' increased less than 5 Pa per hour (see ref. 27). During a creep test, the strain is recorded after application of constant stress. In fatigue or stress sweep tests, a constant or an increasing stress amplitude is applied, respectively. In a typical stress sweep test, the stress amplitude was increased from 0.1 to 1000 Pa (10 points per decade) and each data point was generated from a single oscillation period. As milk gels cannot be rejuvenated by shear, each creep, fatigue or stress sweep curve was acquired on a fresh gel. Identical results were obtained during stress sweep tests using the CC17 Couette geometry or a cone plate geometry, suggesting that no wall slip occurred during gel failure.

2.3 Confocal imaging

Confocal images of the gels were acquired using an inverted microscope, as described elsewhere.[27] When the volume fraction in CMs was varied, the ratio of dry casein to the fluorescent dye (Fast green) was kept constant at 2.4×10^3 (w/w).

3 Results and discussion

3.1 Irreversible yielding during creep and fatigue tests

Fig. 1 displays the creep curves of enzymatic milk gels under different stresses σ_0, applied instantaneously at $t = 0$. In Fig. 1A, γ increases rather slowly at first, and then diverges as the gel completely fails, corresponding to the failure time τ_f. As displayed in the inset of Fig. 1A, τ_f shows a power law relationship with σ_0, $\tau_f \propto \sigma_0^{-\beta}$, with $\beta = 2.0$ (95% confidence interval [1.8; 2.1]). Some results obtained by Zoon[12] during creep tests performed on enzymatic milk gels (pH = 6.65, $T = 30$ °C, aging time = 3.5 h) are also plotted on the graph and yield the same power exponent. It indicates that this exponent value is not sensitive to small variations (*e.g.* the milk origin) and is an inherent property of enzymatic milk gels. The power law relationship between σ_0 and τ_f is reminiscent of the Basquins law of fatigue and is characteristic of an irreversible yielding, which contrasts with the delayed yielding observed for colloidal gels that can be rejuvenated by shear.[8] The waiting time exponent β ranges between 1 and 10 for soft gels, and is related to the damage accumulation rate and the load distribution in the material.[8,28] For 4% w/w acid-induced caseinate gels, an exponent of $\beta = 5.5$ was found during creep measurements.[19] While both gels are made of caseins and exhibit brittle failure, the lower waiting time exponent found for enzymatic milk gels suggests a different mode of failure in comparison with acid-induced caseinate gels.[28]

During the primary part of the creep, $\dot{\gamma}$ first decreases to a minimum value $\dot{\gamma}_{\min}$ and then increases until complete failure of the gel (Fig. 1B). As highlighted by the red line in Fig. 1B, $\dot{\gamma}$ decreases as a power law of time $\dot{\gamma} \propto t^{-0.7}$. Such power law relationship is known as Andrades law and the power exponent is close to 2/3, a value classically found for hard solids, where the primary creep is associated with a succession of plastic deformations.[8] However for some materials, the Andrades law has rather been attributed to their linear properties.[19,29] From the inset of Fig. 1B, the viscoelastic moduli of the enzymatic milk gel in the linear domain roughly follows a power law behavior with the frequency for f below 10^{-2} with $G' \sim G'' \propto f^{0.27}$. At such low frequencies, relevant for creep experiments, $\gamma \propto f^{-\alpha}$ or $\gamma \propto t^{\alpha}$, thus $\dot{\gamma} \propto t^{\alpha-1}$. In the primary part of the creep $\dot{\gamma} \propto t^{-0.7}$, which corresponds well to $\alpha = 0.27$ obtained from the frequency sweep. Thus, the primary creep observed in Fig. 1B may be attributed to the viscoelastic properties of the gel. This assumption can be contrasted with recent results obtained with model colloidal gels that exhibit reversible viscoplastic failure, where varying the linear viscoelasticity of the gels did not affect the value of the exponent ($\dot{\gamma} \propto t^{-0.6}$) for the power law decrease of the shear rate during primary creep.[30]

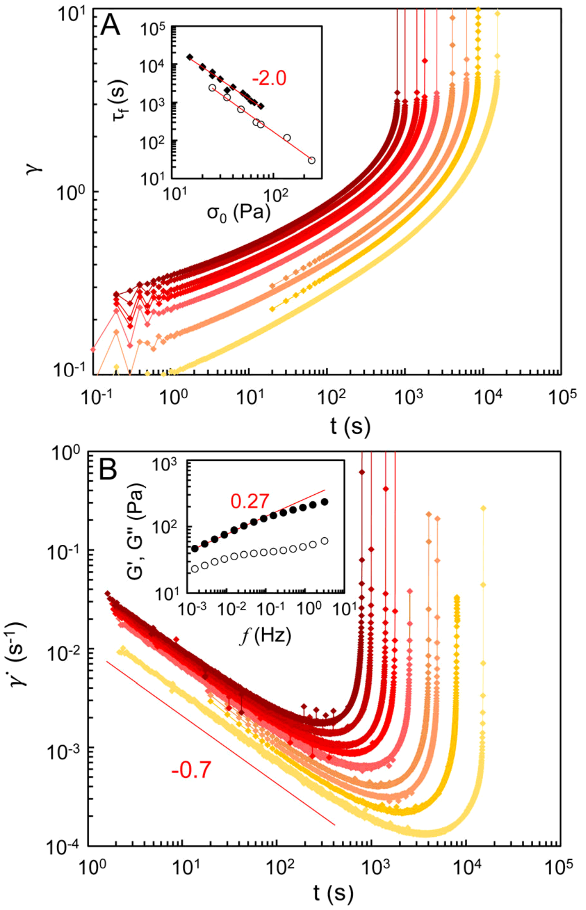

Fig. 1 Creep test of enzymatic milk gel (pH 6.6, $T = 30$ °C). (A) Strain (γ) change during application of various constant stress (σ_0) at $t = 0$. From left to right σ_0 = 75, 65, 55, 50, 40, 30, 25, 20, and 15. Inset displays the time up to failure (τ_f) as a function of σ_0 for the data of the present study (full diamonds) and the data reported by Zoon[12] (open circles). Red lines are the best power law fit $\tau_f \propto \sigma_0^{-\beta}$ with $\beta = 2.0$. (B) Shear rate ($\dot{\gamma}$) evolution corresponding to the creep curves of A). Inset displays the linear viscoelastic moduli G' (full circles) and G'' (open circles) of the enzymatic milk gel as a function of frequency f with $\gamma = 0.01$. The red line is the best power law fit $G' \sim G'' \propto f^{0.27}$ for $f < 1$ Hz.

In the final part of the creep, γ increases and diverges as the gel completely fails. By coupling rheological measurements with high-frequency ultrasonic imaging[19] or light diffraction,[31] it was shown that $\tau_{\min}$, the time corresponding to $\dot{\gamma}_{\min}$, is the moment when cracks nucleate, preceded by a cascade of plastic events. For acid-induced caseinate gels, it was reported that crack nucleation and growth occur at length scales that are lower than 50 μm.[20] For acid-induced caseinate gels, $\tau_{\min}$ linearly scales with τ_f[19] and such relationship, known as the Monkman–Grant relation, has been used to predict failure in hard brittle solids. Leocmach *et al.*[19] have shown that the Monkman–Grant relation

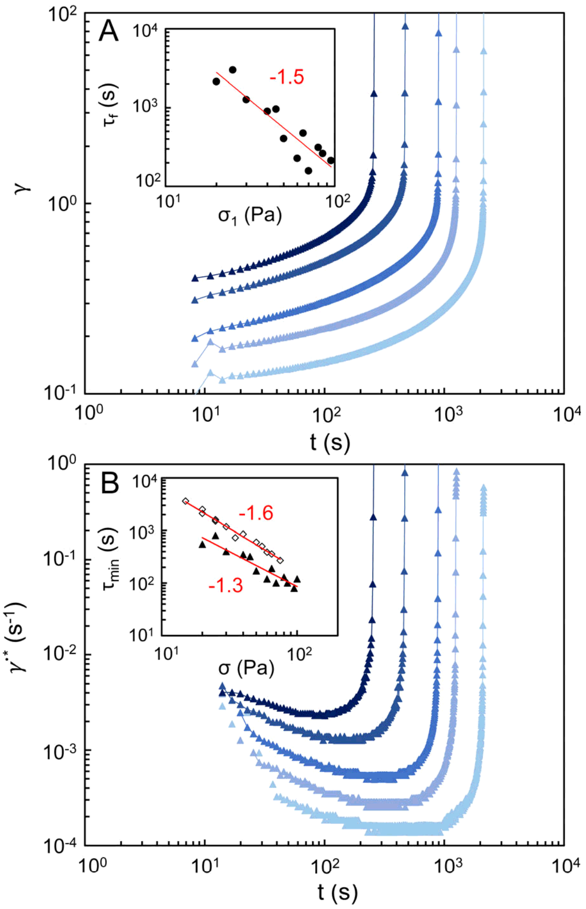

Fig. 2 (A) Fatigue test of the enzymatic milk gel (pH 6.6, T = 30 °C): strain amplitude (γ) change during application of various stress amplitudes (σ_1) at a frequency f = 1 Hz. From left to right σ_1 = 85, 65, 40, 30 and 20 Pa. Inset displays the time up to failure (τ_f) as a function of σ_1. Red line is the best power law fit $\tau_f \propto \sigma^{-\beta}$ with β = 1.5. (B) Apparent shear rate ($\dot{\gamma}$) calculated as the measured rate of shear between two points of the fatigue test displayed in (A). Inset displays the time up to the minimum shear rate (τ_{min}) as a function of the applied stress for creep (open diamonds) and fatigue (full triangles) tests. Red lines are the best power law fit $\tau_{min} \propto \sigma^{-\beta}$ with β = 1.6 and 1.3 for creep and fatigue tests, respectively.

stems from the simple addition of the linear viscoelastic response of acid caseinate gel and fracture growth, with little plasticity in between. By contrast, for the enzymatic milk gel, we observe that τ_{min} rather decreases with a weaker power law of σ_0 than τ_f, namely $\tau_{min} \propto \sigma_0{}^{-1.6}$ (95% confidence interval [1.5; 1.7]), as shown in the inset of Fig. 2B. Thus the ratio $\tau_{min}/\tau_f \propto \sigma_0{}^{0.4}$ decreases with a decrease of the applied stress. This implies that the relative duration of the final part of the creep becomes longer as the applied stress decreases and the total duration of the test increases.

For viscoelastic materials, it was proposed that fracture would occur when the elastic energy concentrates at the tips of the cracks, which implies that the energy stored elastically is higher than the energy dissipated by the viscous flow.[16] For enzymatic milk gels, the loss tangent $\tan\delta$ increases with decreasing frequency[14,27] so that the proportion of the energy dissipated by viscous flow likely depends on the time-scale of the experiment, *i.e.* on the applied stress for creep tests. This would explain the relative increase of the time of the final creep for longer experiments, as the elastic energy would be more efficiently dissipated. For acid-induced caseinate gels, $\tan\delta$ is nearly independent with the frequency $f \in [10^{-4};10^1]$ Hz[14] which is consistent with the observation of the Monkman–Grant relation.

Fig. 2 displays the strain response of the gel under oscillatory shear at a constant stress amplitude σ_1, namely the fatigue test. In comparison with creep tests, the strain at failure γ_f is lower during fatigue as shear is applied in both directions of rotation (Fig. 1A and 2A). As observed for creep rupture, the fatigue failure of enzymatic milk gels also obeys the Basquin's law, with β = 1.5 (inset on Fig. 2A). However, because of the high incertitude over the exponent determination (95% confidence interval [0.9; 2.1]), the waiting exponent obtained during fatigue and creep are not significantly different. While the same range of loads was applied during creep or fatigue tests, the times at failure are about one order of magnitude lower during fatigue. Such a difference might be related to the difference between the timescales involved in the two tests. During creep, the timescale of the test is equal to τ_f with $\tau_f > 1000$ s, while it is equal to 1 s during fatigue at 1 Hz. As mentioned above, it is expected that the proportion of the energy dissipated by viscous flow increases when the load is applied over higher timescales that would slow down damage accumulation and failure. Acid-induced caseinate gels also show shorter failure times during fatigue in comparison with creep. However, the difference of failure time is much smaller as the viscoelastic behavior of the gels is less dependent with respect to the time of the test.

The power law relation between τ_f and σ observed both during creep and fatigue tests, as well as the divergence of the strain during failure are characteristic features of a brittle-like yielding.[8] The yielding under creep for enzymatic milk and acid caseinate gels is very similar, being composed of an Andrade creep regime followed by nucleation and growth of fractures. However, in the case of enzymatic milk gels, the finite lifetime of some bonds, evidenced by the dependence of the loss tangent with the frequency, cannot be neglected and it can slow down fracture growth. Consequently, the Monkman–Grant relation is not verified and viscous dissipation mechanisms must be accounted for to fully capture the creep yielding of enzymatic milk gels.

3.2 Strain-softening and strain-hardening during stress sweep tests

In this section, the gel yielding is characterized by stress sweep tests during which the stress amplitude σ of the oscillations is progressively increased. A typical stress sweep obtained for an enzymatic milk gel is displayed in Fig. 3A. The stress sweep of an acid-induced milk gel, obtained from the same milk, is also depicted for comparison. To better compare the non-linear behavior of samples having different moduli, G' and G'' are normalized by their value in the linear domain, G'_0 and G''_0, respectively. In the linear domain, $\gamma \propto \sigma$ and G' and G'' are independent of σ. At higher σ, G' and G'' increase for the acid-

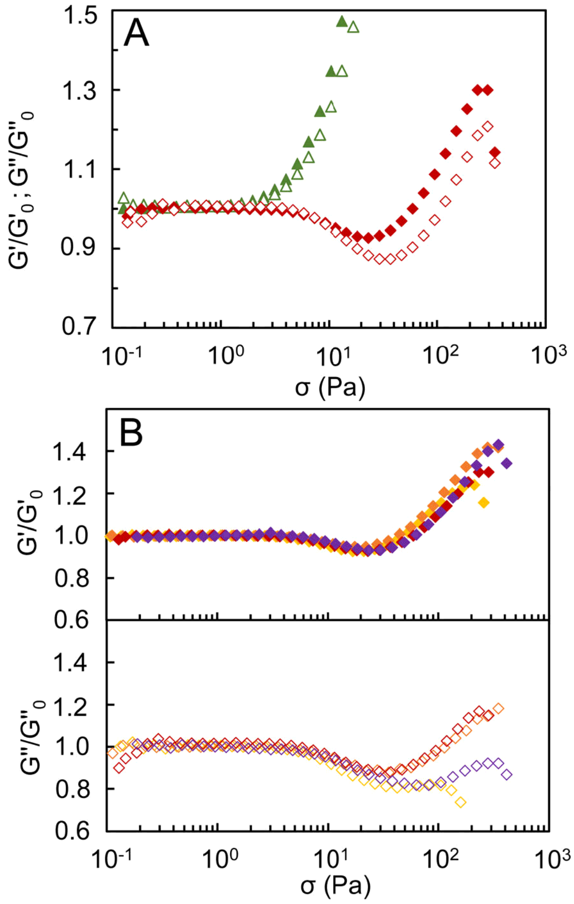

Fig. 3 (A) Stress sweep tests (T = 30 °C, frequency f = 1 Hz) of an enzymatic milk gel (diamonds) and an acid milk gel (triangles): elastic modulus G' (full) and viscous modulus G'' (open) normalized by their value in the linear domains G_0' and G_0'', respectively, as a function of the stress amplitude (σ). Acid milk gel was prepared by adding glucono-delta-lactone powder (1% w/w) to reconstituted skimmed milk and the stress sweep was initiated 20 h after glucono-delta-lactone addition, *i.e.* at a final pH value of ≈4.4. (B) Stress sweep tests of an enzymatic milk gel at different frequencies: G' (full) and G'' (open) as a function of σ. Tested frequencies were f = 0.1 (yellow), 0.5 (orange), 1 (red) and 5 Hz (purple).

induced milk gel, indicating that the material hardens before failure, as reported for acid-induced caseinate gels.[8] The enzymatic milk gel also shows hardening before failure but preceded by softening. This softening, occurring at lower stress amplitudes than the hardening, seems qualitatively different from the softening occurring at higher strain amplitudes than the hardening reported for acid-induced caseinate gels during strain-controlled tests.[21]

This type of non-linear response (softening, then hardening, then failure), has also been reported for networks made of semi-flexible polymer chains,[32,33] for natural rubber latex gels after cyclic straining[34] and for agarose gels under compression.[35] It has been related to the network topology and the inner stress of the gels by Bouzid *et al.*[36] From numerical simulations, they have shown that if gel strands are bent at rest, their release of bending energy at intermediate strains allows to accommodate shear, a phenomenon responsible for the strain-softening during oscillatory shear. This non-linear response preserves the connectivity of the network and is fully reversible, contrary to the softening generally due to connectivity loss or plastic flow observed during the yielding of colloidal gels.[8] Strain-hardening rather originates from the low connectivity of the network, allowing for structure stretching in the direction of shear.[36–38] The amplitude of the strain-hardening is usually small, with the moduli increasing at the most by a factor of 5. For the studied enzymatic milk gel, the moduli vary between 0.9 and 1.3 times their value in the linear domain, indicating that the non-linear behavior of the gel is moderate.

Over the tested range, varying the frequency ($f \in [0.1;5]$ Hz) of the stress sweep had little effect on the strain-hardening or strain-stiffening behavior (Fig. 3B). At f = 0.1 Hz (yellow curve), the amplitude of the hardening appeared to decrease and G'' shows only an overshoot. The decrease of the strain-hardening amplitude at low frequency may result from the relaxation of the stretched strands as the time-scale of the test increases.

To get more insight into the cause of the strain-softening and strain-hardening, we vary the volume fraction of CMs. Since CMs are colloids whose properties highly depend on the physico-chemical state of milk, we need to keep the milk salt equilibria constant.[27] To do so, we concentrate the milk by membrane ultrafiltration. Conversely, we use the ultrafiltration permeate to dilute standard milk and reach lower volume fractions. Fig. 4 displays confocal images of the gel at ϕ_{CM} = 0.05, 0.09 and 0.19. At a low volume fraction, the gel structure appears more heterogeneous, composed of larger strands, larger clusters and larger pores, while the gel at the highest volume fraction has a more homogeneous structure, with reduced pore size. Fig. 5 compares the stress sweep of the resulting gels, with $\phi_{\mathrm{CM}} \in [0.05;0.19]$. The onset of strain-softening occurs at increasing stress for increasing ϕ_{CM}, but at a constant strain ($\gamma \approx 3\%$) as shown in the inset of Fig. 5 and despite appreciable differences regarding gel structures. Therefore, the origin of strain-softening is not in the topology of the network but at a more microscopic level, probably in CM interactions or inner degrees of freedom.

By contrast, increasing ϕ_{CM} has a strong effect on the strain-hardening that occurs at higher strain and decreases in amplitude until complete disappearance at ϕ_{CM} = 0.19. The loss of strain-hardening was also reported at $\phi \approx 0.19$ for colloidal gels made of natural rubber latex particles.[34] At such volume fraction, the gel structure was not fractal anymore as the calculated cluster radius was only 4 times the colloid radius. For the enzymatic milk gel at ϕ = 0.19, assuming a fractal dimension D_{f} = 2.2[39] and an average CMs radius a = 150 nm,[3] the cluster radius would be $R_{\mathrm{c}} = a \times \phi^{\frac{-1}{3-D_{\mathrm{f}}}} = 1.1$ μm, being ≈8 times the CM radius, which is consistent with the finely distributed clusters observed in the confocal microscopy images (Fig. 4C). At ϕ = 0.19, cage effects may also occur because of the decrease of inter-particle distances. The yield of dense colloidal gels has been reported to occur in two steps, corresponding to bond breaking and cage breaking.[40] Cage breaking is identified by the cross-over between G' and G'' during a strain-sweep, which is not accessible for stress-imposed tests as performed here.

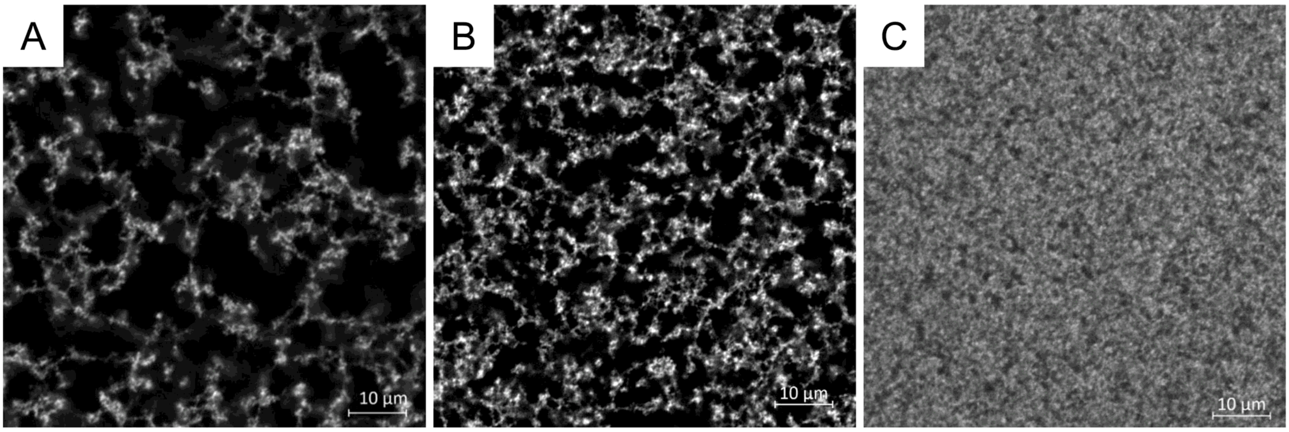

Fig. 4 Confocal microscopy images of enzymatic milk gels with different volume fractions of casein micelles (ϕ_{CM}), with ϕ_{CM} = 0.05 (A), 0.09 (B) and 0.19 (C). The casein network appears in white and the soluble phase of milk appears in black. Representative images are displayed.

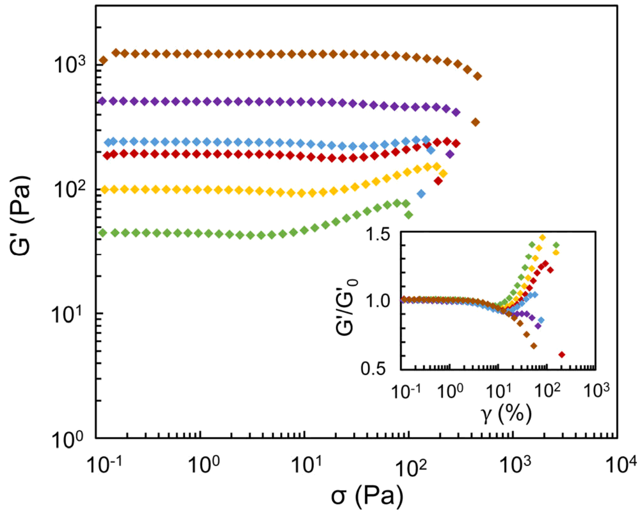

Fig. 5 Stress sweep tests of enzymatic milk gels at different volume fractions of CMs (ϕ_{CM}): elastic modulus (G') as a function of the stress amplitude (σ). From bottom to top: ϕ_{CM} = 0.05, 0.07, 0.09, 0.10, 0.13 and 0.19. Inset displays G'/G'_0 with the elastic modulus G'_0 value in the linear domain (f = 1 Hz) as a function of the strain amplitude (γ).

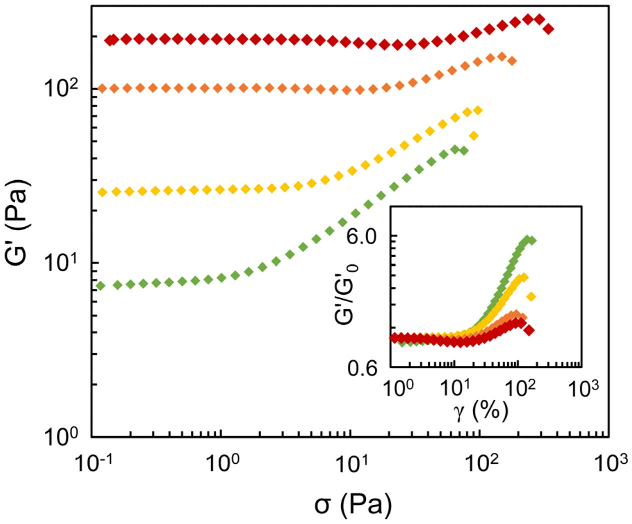

Fig. 6 Stress sweep tests of an enzymatic milk gel (T = 30 °C, frequency f = 1 Hz) initiated at different times after the gelation time (t_{gel}): elastic modulus (G') as a function of the stress amplitude (σ). From bottom to top, $t - t_{gel}$ = 5, 15, 60 and 260 m. Inset displays G'/G'_0 with the elastic modulus G'_0 value in the linear domain (f = 1 Hz) as a function of the strain amplitude (γ).

We can also monitor the non-linearities during the aging of the gel. Fig. 6 displays the stress sweep of enzymatic milk gels initiated at different aging times. Shortly after the gelation time, the enzymatic milk gel displays an intense strain-hardening, up to 5 times the G' value in the linear domain while strain-softening is absent. The fast increase of the viscoelastic moduli at short aging times, resulting from the spontaneous aging of the gel, probably hides the strain-softening, as the gel evolves during the stress sweep. The structure of enzymatic milk gels becomes more heterogeneous during aging,[27] while the potential interaction between colloids remains theoretically constant. In the framework of Bouzid *et al.*,[36] the more homogeneous structure and the higher connectivity at short aging time would be associated with moderate non-linear properties and brittle failure, as the stress localization is reduced. Aging is fast for enzymatic milk gels in comparison with other glassy systems, as it involves micro and mesoscopic rearrangements of the structure that are cooperative and propagate at distances larger than the pore size.[36] Thus, it is likely that successive oscillations may accelerate gel aging, by promoting local relaxations and rearrangements, explaining the high amplitude of hardening at a short aging time.

To give insights into the damage to the gel at a given stress amplitude, we measured its recovery after applying instantaneous oscillatory shear over 10 oscillations at a given stress amplitude σ_1 (Fig. 7A). We observed that after a transient regime, G' reaches a plateau that is lower for larger applied σ_1. In Fig. 7B, the value of G' after recovery is displayed as a function of $\sigma_1/G'_{\text{init}}$, with G'_{init} the elastic modulus of the

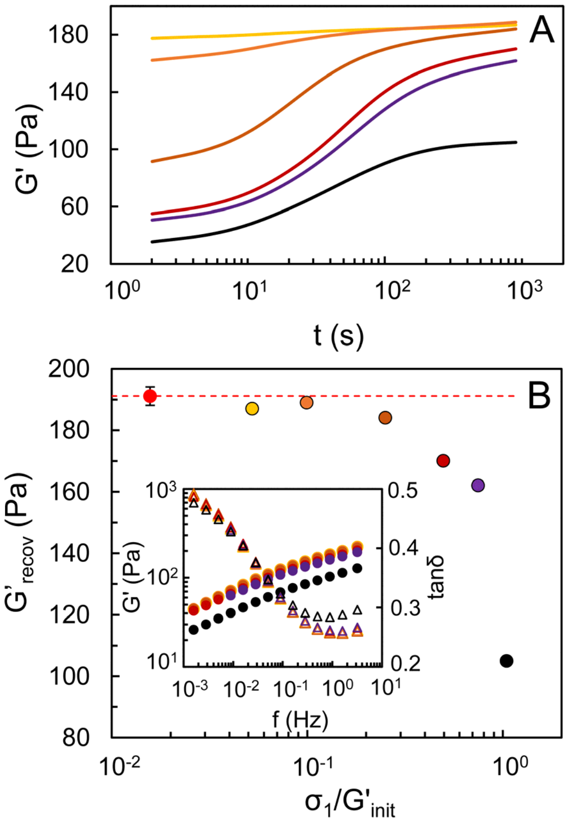

Fig. 7 Enzymatic milk gel (pH 6.6, T = 30 °C) recovery after sudden oscillatory shear. (A) Elastic modulus G' as a function of the recovery time measured under small oscillatory shear (γ = 0.01; f = 1 Hz) after 10 oscillations (f = 1 Hz) with various stress amplitudes σ_1. From top to bottom σ_1 = 10, 19, 48, 95, 143 and 200 Pa. (B) $G'(t$ = 900 s) as a function of $\sigma_1/G'_{\text{init}}$ with $G'_{\text{init}} = 191 \pm 3$ Pa, the initial elastic modulus (γ = 0.01; f = 1 Hz) before shear. G'_{init} is displayed in red. Inset displays a frequency sweep (γ = 0.01) initiated at t = 900 s. The same color code is used as in (A and B).

undamaged gel. At low stress amplitudes $(\sigma_1/G'_{\text{init}} < 0.25)$, the gel fully recovers its initial G' value. Above this limit, the gel recovers a lower fraction of its initial G' with increasing σ_1. We stress that $\sigma_1/G'_{\text{init}} \approx 0.25$ corresponds to $\sigma_1 \approx 50$ Pa, the stress amplitudes at which the gel goes from strain-softening to strain-hardening during the stress sweep. It indicates that the gel is irreversibly damaged during strain-hardening, as expected from strands over-stretching, but softening is reversible. This latter observation rules out a softening of the gel strands by irreversible plastic deformation.[41] Beyond $\sigma_1/G'_{\text{init}} \approx 0.25$, the extent of irreversible damaging is moderate as G' decreases only by $\approx 15\%$ for $\sigma_1/G'_{\text{init}} = 0.75$. Only application of a stress amplitude so that $\sigma_1/G'_{\text{init}} \approx 1$ led to a dramatic decrease of the G' after recovery. Inset in Fig. 7B displays the frequency dependence of G' and $\tan\delta$, determined after recovery. Increasing σ_1 had little effect on the gel viscoelasticity, except for $\sigma_1/G'_{\text{init}} \approx 1$ where a higher viscous-like behavior is observed at high frequency.

Our results compare well with acid-induced caseinate gels that also recover part of their initial modulus value after successive oscillations[20] and their viscoelastic properties after recovery are unaltered up to at least 30% strain.[19] The fact that these gels accumulate damage silently during oscillatory tests, with little impact on the viscoelastic properties till the edge of failure, is consistent with the brittle failure observed during creep.

4 Conclusions

As reported for acid-induced caseinate gels,[8,19] enzymatic milk gels display irreversible and quasi-brittle failure, a feature observed during both continuous and oscillatory shear tests. However, contrary to acid caseinate gels, we evidence additional dissipation during the fracture opening of enzymatic milk gels that we assign to short-lived bonds between colloids. The enzymatic milk gels recover most of its elasticity, even when sheared at stress amplitudes that are close to gel failure. This poor accumulation of irreversible damages is consistent with assumptions of fiber bundle models,[42] where fibers are assumed undamaged until they break. During the stress sweep test, enzymatic milk gels display strain-softening and strain-hardening before failure, which is usually reported for biopolymer gels.[32] Strain-softening is independent of CM volume fraction and gel topology, thus we attribute it to local interactions between CMs. By contrast, strain-hardening is more a property of the network, being smaller when the network is more homogeneous, in agreement with theoretical predictions.[36] Although the softening–hardening–yielding response of enzymatic milk gels seems qualitatively different from the hardening–yielding response of acid-induced caseinate gels, we could not characterize in stress-controlled experiments the unstable softening beyond hardening. Therefore, further strain-controlled studies on enzymatic milk gels are required to understand how the softening before hardening we observe is related to the softening beyond hardening quantified by Keshavarz *et al.* on acid caseinate gels.[21]

From a materials science perspective, our study shows the importance of the nano-scale organisation and the interactions of the building blocks of a gel. The differences between acid-induced and enzymatic gels could appear minute, but they highlight that the topology of the gel network is not always the primary cause of discrepancy in nonlinear mechanical property. As a consequence, one might observe that the micromechanics of fracture opening, *e.g.* the way a single gel strand breaks under stress is altered between the two types of gel because enzymatic milk gels retain the casein micelle structure whereas acid caseinate gels do not. Direct microscopic observation of the failure process would be an interesting path forward to investigate this insight and its consequences on material design.

Enzymatic milk gels are complex, safe and easily prepared systems displaying physical aging and syneresis. The CM properties can also be easily varied by changing the pH or the salt content of milk, providing an interesting system for experimental physicist working with soft matter. Moreover, a better understanding of the failure of these gels is relevant for the dairy industry, as these gels are usually cut during processing or destroyed during consumption. In particular, the effect of the aging time on the non-linear behavior of enzymatic milk gels is highly relevant for the cheese industry, as the gels are cut while aging during cheese manufacture.

Author contributions

Julien Bauland: conceptualization, writing – original draft, data curation, formal analysis, and investigation. Mathieu Leocmach: conceptualization, investigation, formal analysis, writing – review & editing, supervision, and visualization. Marie-Hélène Famelart: conceptualization, investigation, formal analysis, writing – review & editing, and supervision. Thomas Croguennec: conceptualization, investigation, writing – review & editing, supervision, funding acquisition, and project administration.

Conflicts of interest

There are no conflicts to declare.

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