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Chicken Pepsin and Rennet Gels: Internal Bonds, Rheology and Microstructure

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Abstract

The internal bonds created in milk gelation with chicken pepsin were approached by the use of dissociating agents-sodium dodecyl sulfate (SDS), urea, ethylenediamine tetraacetic acid (EDTA) and compared to those with rennet gel. Gels viscoelasticity aspects and microstructure were studied using rheological dynamic non-destructive measurements and Scanning Electron Microscopy (SEM) respectively. Results showed that no significant differences ($p>0.05$) in protein interactions between the two types of gel, with hydrophobic binding dominance in protein gelation. Viscoelasticity and microstructure also showed a similarity in the viscoelastic properties and in milk aggregation micelles mode, obtained by chicken pepsin or rennet.

Keywords: Chicken pepsin; Internal bonds; Viscoelasticity; Microstructure

Introduction

Pepsin is a proteinase, mainly used in food industries. Porcine, ovine, bovine, poultry and aquatic species are its principal sources. In Algeria, coagulant enzyme utilisation relies on importation. However, the increase in its poultry farming from 220 000 tons in 2008 to 300 000 tons in 2011 and the increase in its milk production from 1.5 billion (10^9) litres in 2008 to 2 billion litres on 2012 as related by the Food and Agricultural Organisation [1] were a sufficient and a good encouragement to recover and find ways of using chicken pepsin locally in milk coagulation. Thus, costs of proteinases importation will be reduced. Algerian investors interested in producing this enzyme may contribute to the development of the poultry industry. For this aim, a comparison with the rennet as the reference milk coagulant is necessary to reassure investors of the chicken pepsin feasibility as a coagulant agent. This research objective is the exploitation and assessment of the chicken pepsin properties for its use in the coagulation milk as a rennet substitute.

This study was based on pepsin and rennet gels comparison in order to provide information's on: 1) the nature protein interactions involved in gel chicken pepsin and rennet coagulation by the use of various dissociating agents, 2) the gel viscoelastic aspects, 3) the gel microstructure using Scanning Electron Microscopy (SEM).

Materials and Methods

Skim milk

Medium heat spray-dried skim milk powder (Molochansk Dairy, Ukraine) was reconstituted at 12% (w/vol.) in aqueous $CaCl_2$ (0.01 M) solution and equilibrated over night at 4°C. Sodium azide (0.04% w/vol.) was used to prevent microbial growth.

Rennet

Rennet presented as a commercial bovine powder (MARSCHALL, Rhodia-food, France) with up to 75% of chymosin. It was reconstituted at 1% (w/vol.) to coagulation strength of 100000 Soxhlet Units.

Chicken pepsin

Chicken pepsin extracted from proventriculus (100 g) using 300 mL of a 30 g NaCl - 7 g $NaHCO_3$ per litre solution. After grinding and filtration, the crude extract was activated to pH 2 for 30 minutes, then adjusted to pH 6.6 [2]. The crude pepsin extract had coagulation strength of 13142 Soxhlet Units.

Milk clotting activity

Skim milk (Berridge substrate) coagulation was performed at 30°C in assay tubes by the addition of 1 mL of diluted chicken pepsin or rennet solution to 10 mL of substrate. The coagulation time was determined when the first visible flakes on the moving glass surface appeared. To compare their milk clotting activity, both of chicken pepsin extract and commercial rennet solution were diluted in order to limit the milk clotting time between 5 and 6 minutes [3].

Dissociating agents and calcium chelator

Samples of chicken pepsin or rennet milk gels (8 mL) were dispersed in 32 mL of aqueous dissociating solutions containing the highest concentration of SDS (1 g 100 mL^{-1}) or 6 M urea or 100 mM EDTA as described in Lefebvre-Cases et al. [4]. For the reference sample, 32 mL of distilled water was used instead of the dissociating solution. The mixtures obtained (gels+dissociate agent or gels+distilled water) were quickly homogenized by the use of polytron (PT10-35, Kinematica AG New Zealand) at 30.000 rpm during 45 seconds. After, 1 mL taken from each gel dispersion it was then ultra-centrifuged (Centrikon T-42 K,

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ALC International Srl Italy) at 35000 g and 4°C during 32 minutes. The supernatant (soluble proteins) was kept at 4°C until analysis. The total and soluble proteins were quantified by Lowry's method [5], against a standard curve (DO 750 nm=f [Casein]) (Y=1.2527 X; R^2=0.9906).

Viscoelasticity conditions

Storage modulus G'(Pa), loss modulus G"(Pa) and loss factor δ of pepsin or rennet gels were studied by a dynamic testing technique at frequency 1 Hz in the linear viscoelastic regime (strain<2%), using the measure system cone-plan (4°, 40 mm of diameter) at 20 ± 0.1°C. The rheometer used is StressTech Reologica (Reologica Instruments AB, Scheelevägen 30, 22363 Lund, Sweden).

Scanning Electron Microscopy (SEM)

Pepsin or rennet gels were obtained after 1 h, 9 h and 12 hours of coagulation. Samples preparations were performed according to Attia et al. [6] and then examined with a Philips XL 30 scanning electron microscope (Philips, Limeil Brevannes, France), operating at 30 kV, magnification (X14000) for a scale of 2 µm.

Statistical analysis

Experimental data are presented as means of triplicate measurements and subjected to an analysis of variance using Stat View version 5 (Abacus Concepts™, Berkeley, USA). Differences among means were considered to be significant at p<0.05.

Results and Discussion

Interaction relieved by dissociating agents and calcium chelator

The remarkable effect of SDS on dissociating chicken pepsin and rennet milk gels reflected the important contribution of hydrophobic interactions in the stabilization of the gel structure (Figure 1). The urea action on the proteins dissociation in these two milk gels suggested the presence of a lower level of hydrogen bonds responsible of the structural stability which seems to have the same contribution in every gel network. The action of the EDTA induced dissociation in both of the two milk gels where calcium salts or ionic bonds were complexed [7-9]. This type of interaction seems to be the less abundant. Our data suggest that the rate of protein dissociation is a function of the dissociating agent and the centrifugation test with no significant differences (p>0.05) in protein interactions between the two types of gel. Thus, stabilization of pepsin gel structure seemed to be similar to that of the rennet gel and was supported in the majority by hydrophobic interactions followed by hydrogen and calcic bonds.

Rennet and pepsin gels viscoelasticity variations

Variations of the elastic (storage) modulus G' and the viscous (loss) modulus G" of pepsin gel and rennet gel (Figure 2) progress in the same sense. The elastic modulus (G') fluctuates between 0.28 and 0.74 Pa for pepsin gel and between 0.49 and 1.47 Pa for rennet gel. However, the viscous modulus (G") presents low values with comparison of those of G'. It varies from 0.1 to 0.53 Pa for the pepsin gel and from 0.17 to 0.81 Pa for the rennet gel. On the other hand, the decrease of loss factor δ (<1) showed that elastic character prevails the viscous one for the same gel (G'>G") and between both of gels, noted by the loss factor δ reduction. This parameter is about of 0.60 and 0.55 for pepsin gel and rennet gel respectively. The rennet gel seems to be relatively elastic than the pepsin gel.

According to Vètier et al. [10], conservative module G 'of rennet gel is between 10 and 500 Pascal while the dissipative module G "is between

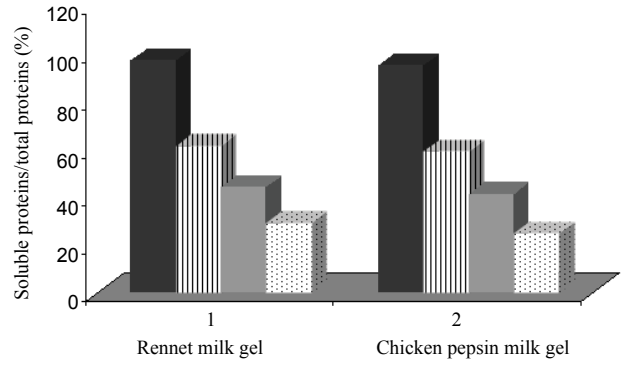

Figure 1: Effect of SDS, UREA and EDTA.

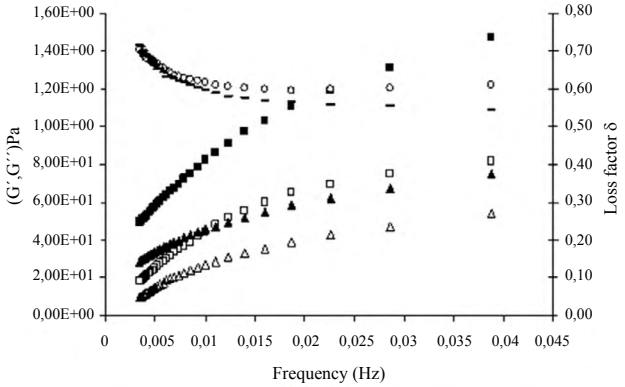

Figure 2: Pepsin and rennet viscoelasticity evolution gels.

10 and 200 Pascal. Indeed, the elastic module (G') which represents permanent connections existing in matrix submitted to deformation is a parameter reflecting the micelles continuous fusion which leads to the increase of the contact area between included particles and the possibility of the incorporation of additional particles to the network gel formed. Thus, the initial loss angle δ is linked to a decrease of the gel viscous character during its deformation. As a consequence, the elastic aspect increases [11]. This stage would allow the creation of a possible new spatial distribution and a structural reorganization creating new interactions (hydrophobic connections, hydrogen and electrostatics bonds) between caseins [12-16].

These interactions which become established in very progressive steps in the rennet gel rennet [9] and probably in the pepsin gel would be responsible in the conservation of their firmness and their compactness.

Microstructure revealed by SEM

At one hour of coagulation, casein particles of pepsin gel and rennet gel (a1, b1) respectively (Figure 3) were connected by zones resulting from fused gels micelles. After 9 hours of coagulation, we noted the passage of the pseudo-network micelles in the chicken pepsin milk gel (a2) and in the rennet milk gel (b2) from a dense structure to a contracted one. At 12 hours of coagulation (a3, b3), similarity between the structure of the two gels was observed where the protein network seemed to have the same numerous cavities of diverse sizes. Micrographs presented by Gastaldi [17], Lagouette [18], Attia et al.

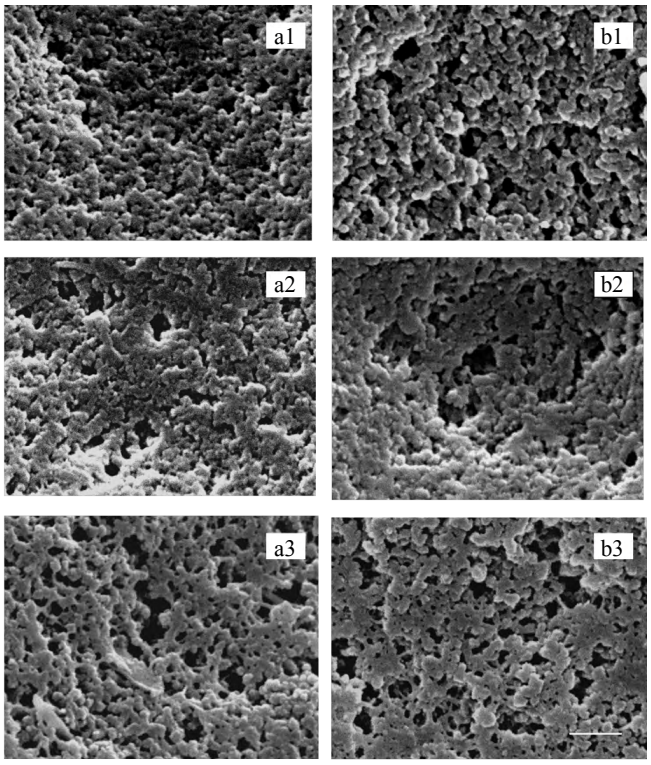

Figure 3: Scanning electron microscopy of (a) pepsin and (b) rennet gels.

[6,19] and Choi et al. [20], for rennet gels obtained after coagulation at pH 6.4-6.6 showed a similitude with the rennet gel microstructure studied mainly at 24 hours of coagulation. These authors describe that the micelles which were perfectly individualized in the pH of the milk begins to gather after rennet coagulation in the spherical particles form. They are of variable sizes and they keep their integrity with protein network formation which presents very numerous cavities of heterogeneous size [21].

Indeed, resemblance between network milk gels structures probably results from the similarity between protein interactions of the chicken pepsin gel and the rennet gel. Therefore the spatial structure seemed to be homogeneous.

Conclusion

Pepsin gel structure seemed to be similar to that of the rennet gel and was supported by the three types of interactions in the following order of abundance: hydrophobic interactions>hydrogen bonds>Ca^{++} bonds. These interactions might reflect the elasticity and the firmness aspects showed by the macrostructure study of viscoelastic properties for both of gels. Also, the microstructure resemblance seemed to be at the origin of protein spatial structure similarity and then between network milk gels structures. This positive results suggest the chicken pepsin use possibility in the milk coagulation as a substitute of rennet and then in the manufacturing of some fresh cheeses. These approaches would contribute in the assessment of an unexploited coagulant enzyme and consequently, in the decrease of rennet and its substitutes import.

Authors Contributions

Ferial Aziza Benyahia-Krid has provided the research design, participated in the research analysis and has draft and revised the paper. Ouarda Aissaoui-Zitoun has participated in the research analysis and has and discussed the results. Halima Boughellout has participated in the research analysis and has draft the paper. Faiza Adoui has participated in the research analysis and has draft the paper. El Hocine Siar has participated in the research analysis. Abdellah Zikiou has participated in the research analysis. Attia Hamadi has provided the research design and participated in rheological analysis. Mohamed Nasser Eddine Zidoune has provided the research design and revised the paper.

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