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Association of chymosin with caseins in solution

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Summary

The association of chymosin with para-κ-casein was studied in a solution of κ-casein in imidazole buffer, with or without α_s- or β-casein being added. Standard conditions were pH 6.3, 30 °C and ionic strength 0.04 *M*. Association was determined by removing the coagulum by centrifugation and determining the activity of chymosin in the supernatant.

The association of chymosin with para-κ-casein was decreased by adding α_s- or β-casein and was increased upon dilution with imidazole buffer. Competition between chymosin and α_s- or β-casein for association with para-κ-casein is offered as an explanation for these results. The association was slightly lower at 10 °C than at 40 °C and decreased with increasing ionic strength or pH. Presumably, hydrophobic interactions as well as electrostatic forces are involved.

Keywords: chymosin; rennet; (para-κ-)casein; association; adsorption; proteolysis; cheese ripening

1 Introduction

During cheese making, part of the chymosin is retained in the drained curd. During cheese ripening, this chymosin hydrolyses the caseins in the cheese. The peptides formed include bitter peptides. Subsequently, the proteases and peptidases of starter bacteria may hydrolyse the peptides into smaller non-bitter peptides and amino acids. These processes contribute to the taste of cheese. Chymosin is therefore an essential factor in cheese ripening (1).

An even distribution of chymosin over the aqueous phases in milk and curd during cheese manufacture would lead to about 5% of the added chymosin being retained in the curd. However, Stadhouders and Hup (1) found this to be about 15% in Gouda cheese. They also showed that by decreasing the pH of the cheese milk or the scalding temperature, the amount of retained chymosin increased.

By using artificial micelle milk, the composition of the casein fraction was found to be an important factor in chymosin retention (De Roos et al., unpublished results). The retention increased when the content of κ-casein in casein fractions increased, and chymosin retention was stronger in case of micelles

composed of β- and κ-casein than in case of micelles composed of α_s- and κ-casein.

Chymosin may be retained in the curd by association with paracasein. The aim of this study was to achieve a better understanding of the mechanism of this association. For that purpose, the interaction between chymosin and several caseins was investigated in a model system. To make this model as simple as possible, caseins were in a dissolved state. Factors investigated were contact time between chymosin and casein, casein and chymosin concentrations, composition of the casein mixture, temperature, ionic strength and pH.

2 Materials and methods

Caseins were dissolved in 0.05 *M* imidazole-HCl buffer of pH 6.3 and ionic strength 0.04 *M* (2). pH 6.3 is a common pH at the start of pressing of curd in Gouda cheese manufacture (3). The concentration of the individual caseins was calculated from the casein content (87% w/w by Kjeldahl analysis) and the composition of the casein preparations used (Sigma Chemical Company, St. Louis, USA). The casein in the κ-casein preparation contained 94.6% κ- and 5.4% α_s-casein, the α_s-casein preparation 88.5% α_s- and 11.5% β-casein and the β-casein preparation 91.2% β- and 8.8% α_s-casein (w/w; estimated from polyacryl-amide-gel electrophoresis and densitometry of the resulting gels). Chymosin (lyophilized powder, 94 units/mg; Sigma Chemical Company) was dissolved in the same buffer as casein. In this buffer, the chymosin activity did not decrease during the experiments.

A 1 ml volume of κ-casein solution (imidazole-HCl buffer for the blank) was mixed with 2 ml of chymosin solution. Concentrations of caseins and chymosin are mentioned later. The mixture was kept stirred at 30 °C. High concentrations of chymosin were used (by a factor 300 to 3000 times higher than in cheese-making), and para-κ-casein coagulated within a few seconds. At 0.5, 2 and 4 min after mixing (contact time), a sample was centrifuged for 1 min at 11000 *g* in an Eppendorf centrifuge to remove the coagulum containing the associated chymosin. In the supernatant, the chymosin activity was determined by the Berridge flocculation test (4): *x* ml was added to a polystyrene conical tube containing 0.15 ml reconstituted whey (no residual chymosin activity; Borculo Whey Products, Borculo, The Netherlands) and 0.5 ml 0.2 *M* $CaCl_2$; water was added to give 5 ml. The whey was used to prevent inactivation of chymosin during storage of the sample at about –22 °C (Geurts, unpublished results). A solution of 20 g low-heat skim milk powder in 100 g water (double-concentration reconstituted skim milk (5), stirred at 45 °C for 1 h and stored at 4 °C overnight), the conical tube containing the sample, and a 25 ml beaker with a glass rod were warmed to 30 °C. Then, 5 ml of the milk was added to the sample, mixed, poured into the beaker and kept at 30 °C. The time after which the first flocs became visible was taken as the flocculation time (t_f).

The percentage of associated chymosin (*P*) was calculated using the rule of

Storch and Segelcke (6), which states that the flocculation time is inversely proportional to the chymosin concentration: $P = [(t_{fs} - t_{fb}) / t_{fs}] \cdot 100$, where t_{fs} and t_{fb} are the flocculation times for supernatant and blank, respectively. The maximum chymosin association at a certain chymosin concentration, reached immediately after hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein to form para-κ-casein, was determined by extrapolating the percentages of association after 0.5, 2 and 4 min of contact to reach time zero, using linear regression.

For the calculation of the concentration of chymosin (C) it was assumed that the chymosin concentration in a commercial calf rennet of 10800 Soxhlet units was 13.3 μ*M* (6). 4000-Fold dilution of this rennet was found to lead to a flocculation time of about 300 s. As a standard throughout the experiments a rennet solution guaranteed to contain 9660 Soxhlet units (i.e., 11.9 μ*M* chymosin by using the above-mentioned assumption) (RIKILT-DLO, Wageningen, The Netherlands) was diluted 3578-fold to achieve a flocculation time (t_{fg}) of about 300 s. C was then calculated on the basis of the rule of Storch and Segelcke: $C = (11.9/3578) \cdot (t_{fg}/t_{fb}) \cdot D$, where C is in μ*M*, and D is the dilution factor for the samples. From P, C and the concentration of casein, the association and the chymosin equilibrium concentration can then easily be calculated. Subsequently, isotherms can be made. No correction was made for the volume occupied by caseins; for the highest κ-casein concentration applied (i.e., 15 g/l) the calculated association therefore might be up to 3% too low. Throughout this publication, the association is expressed relative to para-κ-casein, because chymosin appeared to associate mainly with para-κ-casein (7).

To estimate proteolysis, the protein concentration in the supernatants was determined by the Biuret method (2). Absorbance was measured at 20 °C and 540 nm. Bovine serum albumin (Sigma Chemical Company) was used as a reference.

Unless stated otherwise, the experimental conditions were pH 6.3, 30 °C and ionic strength 0.04 *M*.

3 Results and discussion

3.1 Contact time

Figure 1 shows the association of chymosin with para-κ-casein as a function of time, for some chymosin/κ-casein ratios. The association immediately reached a maximum, followed by dissociation. The higher the ratio of chymosin to casein, the faster the dissociation. The dissociation and the increase in protein content of the supernatant were faster at 40 °C than at 10 °C (Figure 4C). A decreasing stability of the para-κ-casein precipitate during some prolonged incubations was also noticed (i.e., the precipitate formed a weaker pellet and was hard to fully separate from the supernatant). Obviously, proteolysis is involved, and this is a complicating factor in determining association. Very high ratios of chymosin to casein and/or optimal conditions for proteolysis may even result in ex-

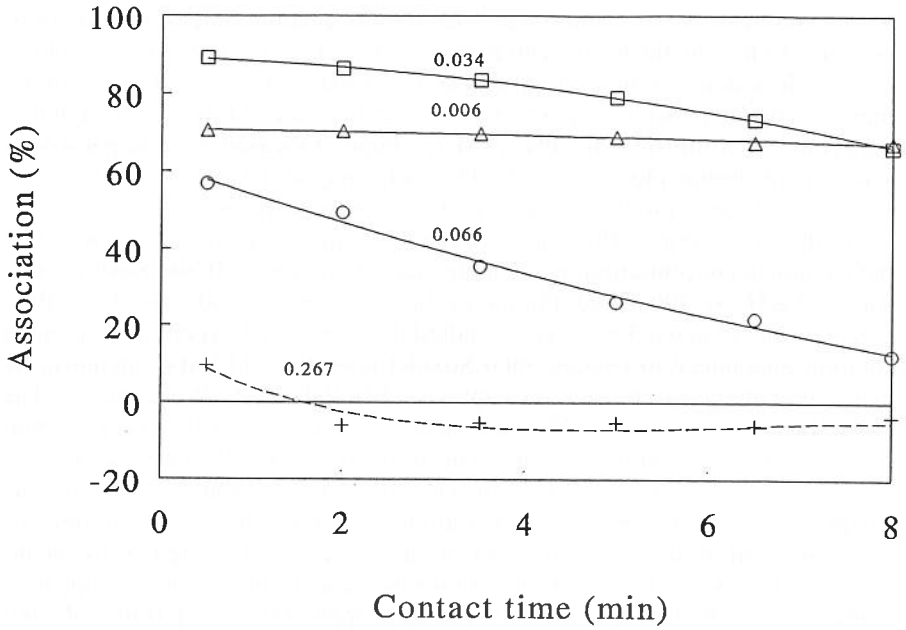


Figure 1. Association of chymosin with para-κ-casein (in % of total chymosin) as a function of time after addition of chymosin to solutions of κ-casein. pH 6.3, 30 °C, ionic strength 0.06 *M* (20 m*M* NaCl added). Concentrations of κ-casein (g/l) and chymosin (μ*M*): 3.3 and 5.71 (□); 1.0 and 0.31 (△); 1.0 and 3.37 (○); 0.15 and 2.05 (+), respectively. The ratio of chymosin to κ-casein (mol/mol) is indicated.

cessive dissociation, so that any determination of association becomes impossible. In the experiments discussed in the following sections, care was taken to avoid such excessive dissociation.

The association of chymosin with para-κ-casein had to be determined immediately after hydrolysis of the Phe_{105}-Met_{106} bond of κ-casein to form para-κ-casein. The above mentioned dissociation and varying rates of proteolysis were, therefore, the reasons to estimate the maximum association by extrapolating the percentages of association to time zero (see also Section 2).

3.2 Casein composition and casein concentration

To further investigate the effect of casein composition as mentioned in the introduction, small amounts of α_s- or β-casein were added to κ-casein solutions. α_s- and β-casein each clearly suppressed the association, α_s-casein having the stronger effect (Figures 2A and 3). Additional α_s-casein in the κ-casein solution further decreased the association, though to a decreasing extent (Figure 2B). Similar results were found at 10 and 40 °C (Figures 4A and 4B). Because of the impurity of the κ- and β-casein preparations, the solutions with added β-casein

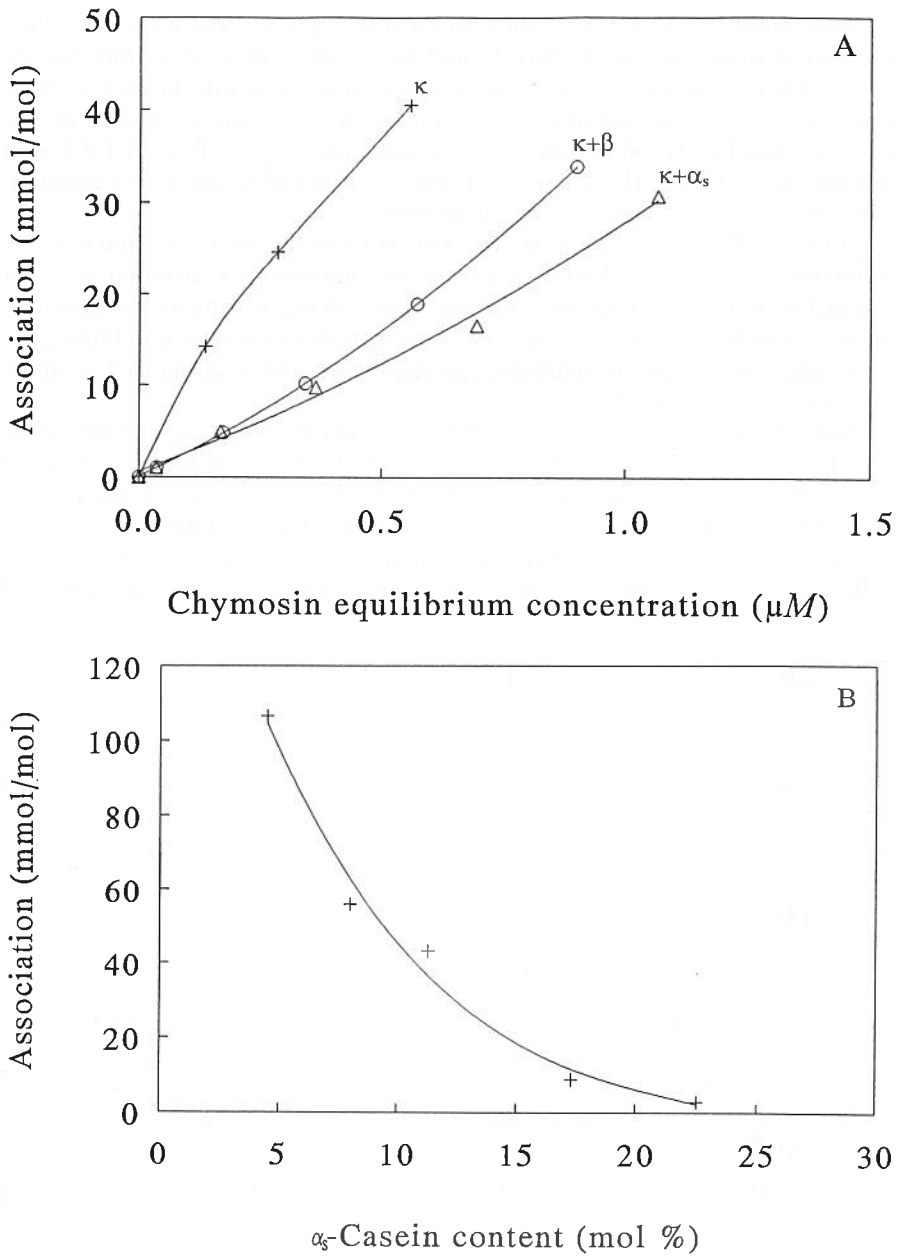


Figure 2. Effect of α_s- or β-casein addition on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in a solution of 1.0 g κ-casein/l. pH 6.3, 30 °C, ionic strength 0.04 *M*. (A) κ-casein (+); 0.05 g α_s-casein/l added (△); 0.05 g β-casein/l added (○). (B) Association at 3 μ*M* chymosin equilibrium concentration, estimated by linear regression of association curves, as a function of the α_s-casein content of the casein fraction.

contained more α_s- (5.6% w/w) than β-casein (4.5% w/w). Therefore, the effect of added β-casein may be (partly) through the action of α_s-casein rather than β-casein. These results may explain the results with artificial micelle milk as mentioned in the introduction and the low chymosin association found in cheese (i.e., 0.1 mmol/mol) and in milk (i.e., 1.4 mmol/mol; α_{s1}:α_{s2}:β:κ = 4:1:4:1.6 on a molar basis, 25 °C, pH 5.0, ionic strength 120 m*M* and κ-casein concentration 130 μ*M* or 2.5 g/l) (Geurts, unpublished results).

At 11.3 mol% α_s-casein it took more than 0.5 min for the association to reach a maximum. Above 17 mol% α_s-casein, the maximum association was not reached within 4 min. Therefore, the true values of the maximum association at these α_s-casein concentrations may be slightly higher than shown in Figure 2B. This effect may be due to inhibited aggregation of para-κ-casein as a result of the presence of α_s-casein (8).

Diluting a solution of κ-casein (with or without added α_s-casein) with imidazole-HCl buffer resulted in a higher association (Figure 3). In the case of added α_s-casein about 13% α_s-casein was present; again, as mentioned in the discussion of Figure 2B, no maximum in association was found within 4 min. An increase in association as a result of dilution has also been found in artificial micelle milk (de Roos, unpublished results). Furthermore, in a very concentrated

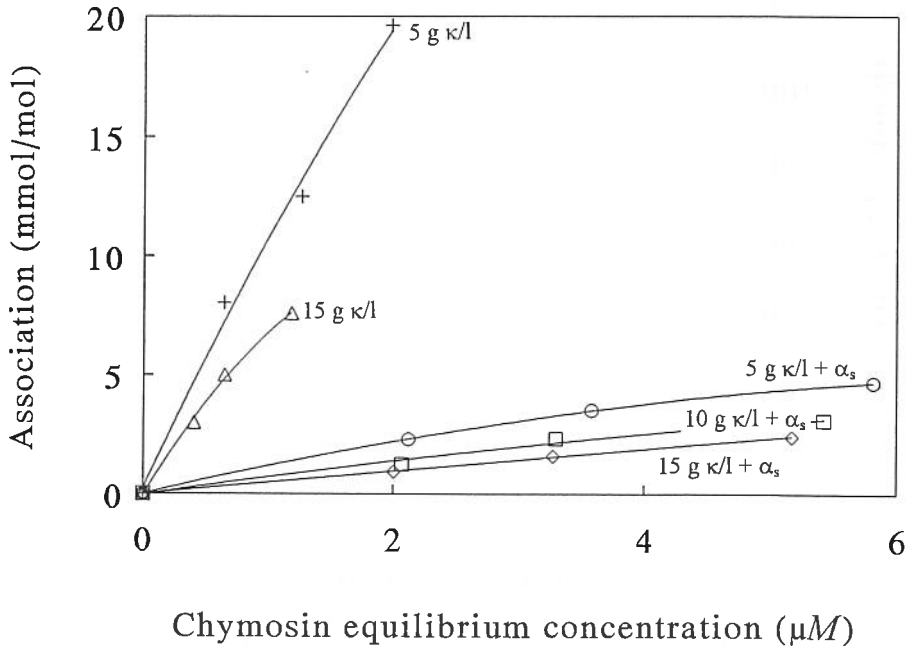


Figure 3. Effect of casein concentration (indicated near the curves) on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in solutions of κ-casein, with or without α_s-casein (κ:α_s 10:1 mass ratio) added. pH 6.7, 30 °C, ionic strength 0.04 *M*.

casein system like cheese an association of 0.1 mmol/mol was found and for milk, the diluted form, 1.4 mmol/mol (see above). Also in soya oil emulsions with κ-casein as an emulsifier, the association of chymosin with para-κ-casein increased by diluting the emulsion (7).

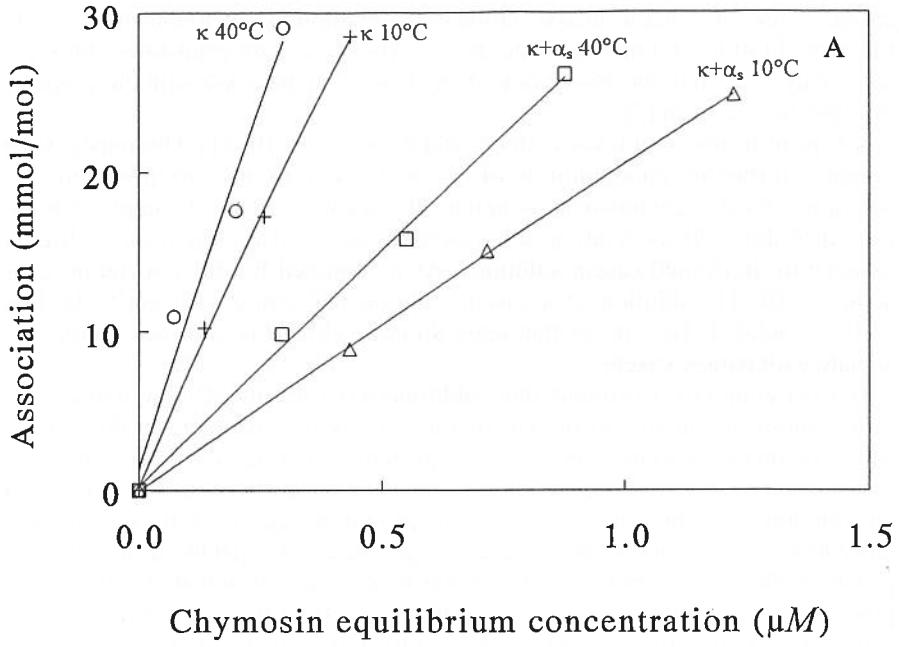
κ-Casein forms complexes with α_s- and β-casein (9, 10, 11). Obviously, such complex formation upon addition of α_s- or β-casein to a κ-casein solution resulted in a weaker chymosin association. The negative effect of complex formation, including self-association of κ-casein (6, 12), is also clear from the higher association in diluted casein solutions. As determined by the association constant (9, 10, 11), dilution of a casein solution will cause κ-casein to be less (self-)associated. This means that upon dilution, chymosin can increasingly associate with para-κ-casein.

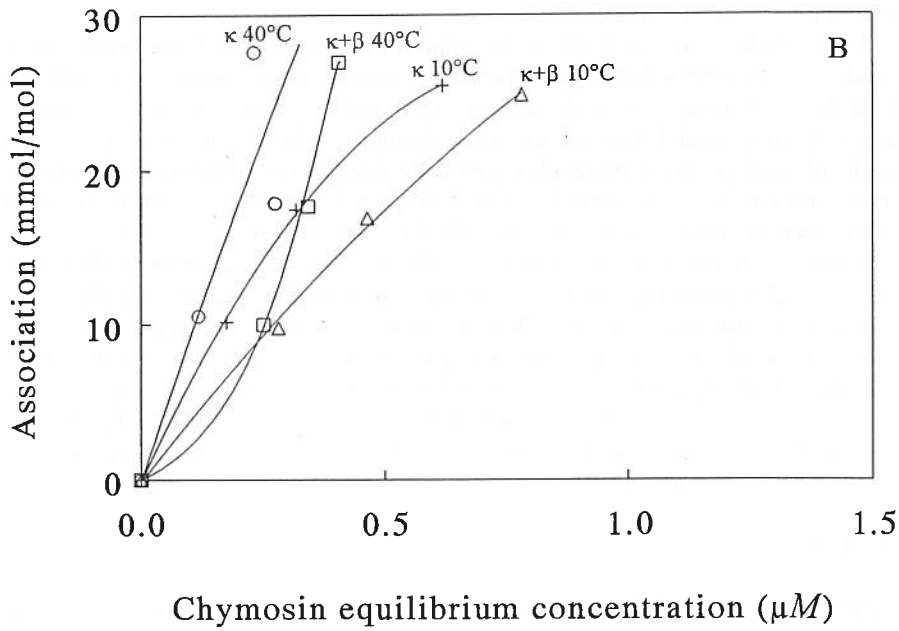
Garnier et al. (13, 14) found upon addition of α_s-casein or β-casein to a κ-casein solution no change in the enzymatic activity of chymosin on the Phe_{105}-Met_{106}-bond of κ-casein, although fewer protons were released. They concluded that α_s- and β-caseins associate with κ-casein on a site different from the site of enzyme binding, but close to the ester bond hydrolysed by chymosin. Binding close to this ester bond would decrease the dissociation constant of the carboxyl group of Phe_{105} of para-κ-casein, leaving it in a more protonated form. Other possible explanations are an increased buffering effect from added α_s- or β-casein and a totally different conformation of the C-terminal end of para-κ-casein, thereby interacting with α_s- or β-casein molecules. Therefore, complex formation between caseins may have caused steric hindrance or different electrostatic interactions, resulting in decreased association of chymosin with the C-terminal end of para-κ-casein.

Kumosinski et al. used molecular modelling to construct three-dimensional models for a casein submicelle consisting of one κ-casein, four α_{s1}-casein B and four β-casein molecules in the absence (15) and presence (16) of water molecules. In their model, two α_{s1}-casein B dimers would interact with a κ-casein molecule and β-casein molecules would be docked into massive hydrophobic areas of the α_s-κ-casein complex. These proposed structures would suggest that interaction of one α_{s1}-casein dimer with the so-called 'front-leg' (residues 20-34) of κ-casein can sterically interfere with the binding of chymosin. However, this in itself cannot explain the enormous effect of α_s-casein on the chymosin association with κ-casein; only 20 mol% α_s-casein sufficed to reduce the association to nearly zero (Figure 2B). Since κ-casein molecules in solution can associate to form structures resembling detergent micelles (12), interaction of one α_s-casein molecule with one κ-casein molecule may also have caused other κ-casein molecules of the same κ-casein micelle to be sterically hindered from associating with chymosin.

3.3 Temperature

Chymosin association in solutions containing κ-casein, with or without α_s- or


30
20
10
0
0.0
0.5
1.0
1.5
Association (mmol/mol)
κ 40°C
κ 10°C
κ+α$_s$ 40°C
κ+α$_s$ 10°C
A
Chymosin equilibrium concentration (μM)


30
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Association (mmol/mol)
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κ 10°C
κ+β 10°C
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Chymosin equilibrium concentration (μM)

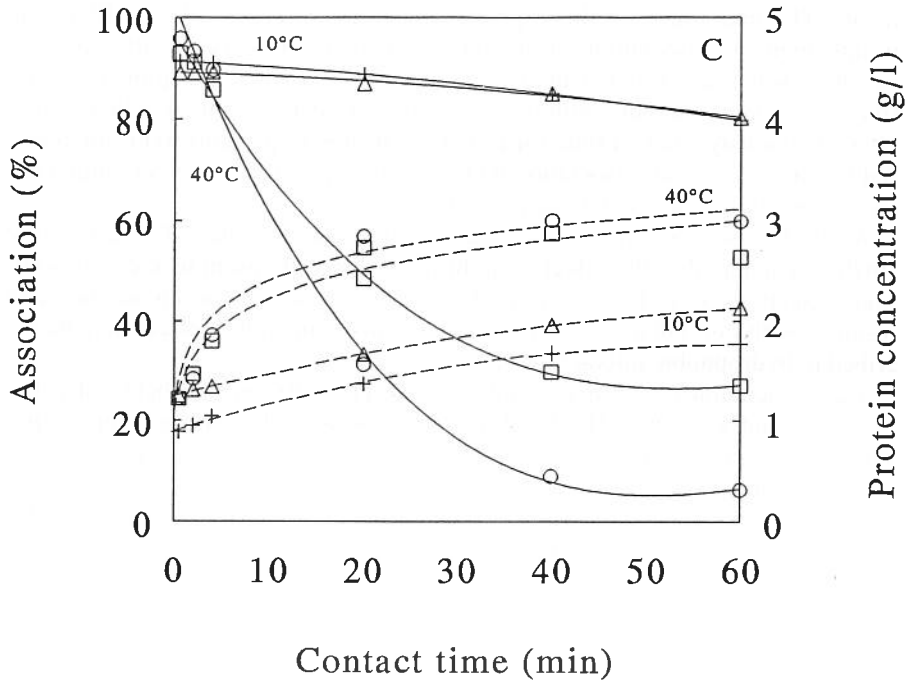

Figure 4. Association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) at 10 and 40°C in solutions of 5 g κ-casein/l, with or without 0.25 g/l added α_s-casein (A) or β-casein (B). (C) Association of chymosin with para-κ-casein (% of total chymosin) and protein concentration in supernatants (broken lines) as a function of time after addition of chymosin to a solution of 5 g κ-casein/l with or without 0.25 g β-casein/l added.
pH 6.3, ionic strength 0.04 *M*. κ-casein at 10°C (+) and 40°C (○), respectively; κ- and α_s- or β-casein at 10°C (△) and 40°C (□), respectively.

β-casein being added, was determined at 10 and 40°C. Because of the impurity of the preparations used, the final α_s-casein concentration in the β-κ-casein solution was 0.05 g/l higher than the β-casein concentration. The casein and chymosin solutions were kept for at least 30 min at the measuring temperature before mixing. Slower or inhibited precipitation of para-κ-casein as a result of weaker hydrophobic interactions at 10°C did not appear to affect the results, since no additional coagulum was formed in the 10°C supernatant after warming it to 40°C. At 40°C the association was higher than at 10°C for all solutions (Figures 4A and 4.B). This seems to contradict the effects of a varying scalding temperature in cheese manufacture, reported by Stadhouders and Hup (1). De Roos et al. (7) also found increasing chymosin association with decreasing temperature in soya oil emulsions with κ-casein as an emulsifier. The discrepancy between this earlier work and the present results may be ascribed to differences in contact times applied, i.e., about 1 h and 20 min for Stadhouders and Hup, and De Roos et al., respectively, and extrapolated to 0 min in the present experi-

ments. The importance of this aspect is illustrated in Figure 4C. At 40 °C, the initial chymosin association was higher than at 10 °C. However, at 40 °C the dissociation was much faster than at 10 °C, probably due to higher proteolytic activity (6, 17); after about 3 min the association at 40 °C became less than that at 10 °C. Obviously, contact time (or rather the degree of proteolysis) is an essential factor in chymosin association with caseins, especially at the very high chymosin concentrations used in the present study.

At 10 °C, association was less than at 40 °C, though the differences were small as compared to the effect of addition of α_s- or β-casein to κ-casein solutions (Section 3.2). Chymosin preferentially cleaves between amino acid residues with non-polar side-chains (18), so this temperature effect may be ascribed to hydrophobic interactions.

The association between α_s- and κ-casein (19, 20), and probably also between β- and κ-casein (21, 22, 23), decreases with decreasing temperature. With κ-casein becoming less (self-)associated, chymosin can increasingly associate with para-κ-casein. This may partly have compensated for the temperature effect on chymosin association (see also effect of dilution, Section 3.2). Hydrophobic interactions may thus have played a more important role than one would conclude by considering only the data presented in Figure 4.

3.4 Ionic strength and pH

Ionic strength was varied by adding NaCl to the casein and chymosin solutions. To determine the association at low ionic strengths, in this experiment a 0.01 *M* imidazole-HCl buffer (I = 8 m*M*) was used. The association decreased with increasing ionic strength (Figure 5). This shows that charged groups were involved in chymosin association.

This can also be concluded from the influence of pH. Increasing the pH from 4.7 (isoelectric pH of chymosin) to 7 [where para-κ-casein is still positively charged (24)] will render chymosin more negative and para-κ-casein less positive. Association was estimated in solutions of 5 g κ-casein/l with or without 0.25 g α_s-casein/l added, at pH 6.3 and 6.7. At 0.5 μ*M* chymosin equilibrium concentration and 30 °C, the chymosin associations were about 13 and 44 mmol chymosin/mol para-κ-casein, respectively, at pH 6.3 (Figure 4A), and 0.4 and 5.0 mmol/mol, respectively, at pH 6.7 (Figure 3). Obviously, chymosin association decreased with increasing pH. Ionic strength and pH seemed to affect chymosin association more strongly than temperature, in the ranges studied. Similar effects of ionic strength and/or pH were found in emulsions containing κ-casein adsorbed onto soya oil droplets (7), in casein suspensions (25) and in artificial micelle milk (26).

The ionic strength and the pH may have affected, among others, the interactions between chymosin and the C-terminal end of para-κ-casein. Besides being affected by the pH, the dissociation of the carboxyl group of Phe-105 of para-κ-casein may be decreased by addition of α_s- or β-casein to a κ-casein solution

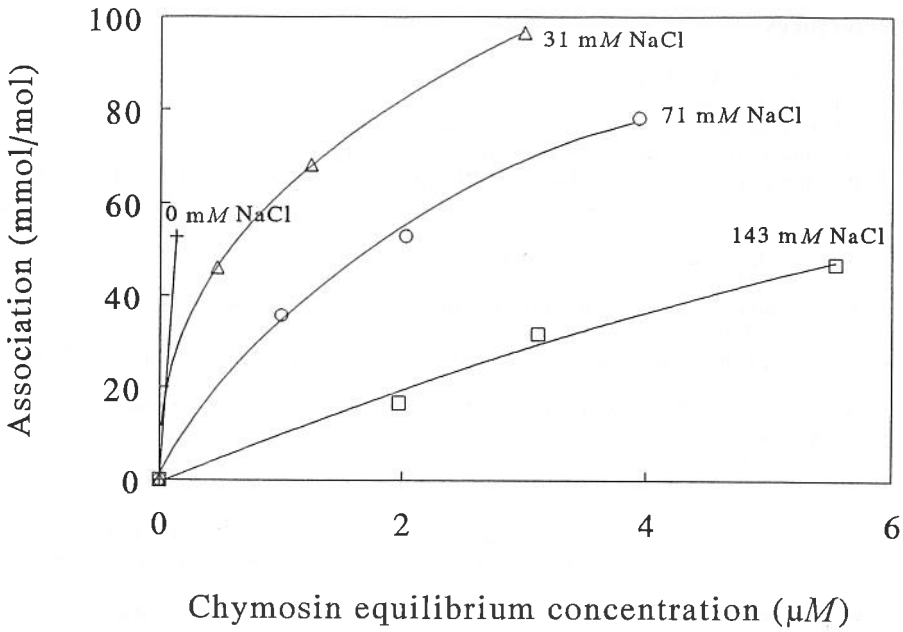

Figure 5. Effect of ionic strength on the association of chymosin with para-κ-casein (mmol chymosin/mol para-κ-casein) in a solution of 1.0 g κ-casein/l in 0.01 *M* imidazole-HCl buffer. pH 6.3, 30 °C. Amount of NaCl added is indicated. $I = 8$ plus amount of NaCl added (m*M*).

(13, 14; see also Section 3.2). Furthermore, Dalgleish (24) concluded that amino acid residues 98-102 of κ-casein (His-Pro-His-Pro-His) are important for chymosin binding. This fragment contains the only three histidine residues in κ-casein. Histidine is the only amino acid of which the pK_a of the side group (6.00) is between pH 5 and 7 (27). The strong association at low ionic strength, i.e. at a thick electrical double layer ($1/\kappa \approx 3.4$ nm at $I = 8$ m*M*), suggests that the three histidine residues are all involved in chymosin association, possibly acting as one positive charge. At higher ionic strength, hence at a thinner electrical double layer (0.8 nm at $I = 151$ m*M*), the histidine residues will increasingly act as separate charges. His-102 is then probably essential (28). Electrostatic interaction between κ-casein and negatively charged κ-carrageenan was also suggested to involve positive charges (including the three histidine residues) on κ-casein. However, at pH 6.7, the latter interaction increased with increasing ionic strength up to 0.2 *M* (29).

Acknowledgments

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