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Influence of ethanol on the rennet-induced coagulation of milk

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The influence of ethanol on the rennet-induced coagulation of milk was studied to investigate potential synergistic effects of these two mechanisms of destabilisation on the casein micelles. Addition of 5% (v/v) ethanol reduced the rennet coagulation time (RCT) of milk, whereas higher levels of ethanol (10–20%, v/v) progressively increased RCT. The temperature at which milk was coagulable by rennet decreased with increasing ethanol content of the milk. The primary stage of rennet coagulation, i.e., the enzymatic hydrolysis of κ-casein, was progressively slowed with increasing ethanol content (5–20%, v/v), possibly due to ethanol-induced conformational changes in the enzyme molecule. The secondary stage of rennet coagulation, i.e., the aggregation of κ-casein-depleted micelles, was enhanced in the presence of 5–15% ethanol, the effect being largest at 5% ethanol. Enhanced aggregation of micelles is probably due to an ethanol-induced decrease in inter-micellar steric repulsion. These results indicate an interrelationship between the effects of ethanol and chymosin on the casein micelles in milk, which may have interesting implications for properties of dairy products.

Keywords: Milk, casein micelles, ethanol, rennet, coagulation.

Caseins, which amount for *ca.* 80% of the proteins in milk, exist as large colloidal aggregates known as casein micelles. It is universally accepted that a diffuse layer of flexible hydrophilic polypeptide chains, principally the C-terminal region of κ-casein, sterically stabilises the casein micelles. This so-called 'hairy layer' presents a virtually-impregnable barrier against aggregation, unless removed or neutralised (Walstra, 1979, 1990; Holt, 1992; Holt & Horne, 1996). Consequently, any process or environmental factor which eliminates the stabilising effect of κ-casein markedly reduces the colloidal stability of casein micelles. General aspects of the colloidal stability and properties of casein micelles have been reviewed comprehensively by Holt (1992), Rollema (1992), Dalgleish (1998), Creamer et al. (1998) and De Kruif & Holt (2003).

Enzymatic hydrolysis of κ-casein, addition of ethanol, exposure to a high temperature (>120 °C) or acidification are the principal factors that affect the colloidal stability of casein micelles. Enzymatic hydrolysis of κ-casein reduces the steric stabilisation of the micelles, as well as inter-micellar electrostatic repulsion, resulting in the coagulation of milk (see Fox et al. 1996). Exposure to a high temperature causes, amongst other changes, the dissociation of κ-casein from the micelle surface (see O'Connell & Fox, 2003), while acidification reduces protein charge and hydration (see Lucey & Singh, 2003). Addition of ethanol reduces the dielectric constant of milk, which causes the collapse of the protruding C-terminal region of κ-casein, with a concomitant reduction in micellar stability (see Horne, 2003). The effects of enzymatic hydrolysis of κ-casein, addition of ethanol, heat treatment or acidification on the colloidal stability of casein micelles have been reviewed by Hyslop (2003), Horne (2003), O'Connell & Fox (2003) or Lucey & Singh (2003), respectively. Also, the interrelationship between some of these factors which reduce the colloidal stability of casein micelles has been investigated, as summarised in Table 1. The influence of ethanol on the stability of casein micelles to rennet-induced coagulation has not yet been investigated, but may offer novel means for altering the properties of rennet-induced milk gels. The results of experiments on the influence of ethanol on the rennet-induced coagulation of milk are reported in this communication.

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Table 1. Overview of studies reporting interrelationship between factors that destabilise casein micelles in milk

	Heat	Rennet	Acid	Ethanol
Heat stability	—	Fox & Hearn, 1978	Rose, 1961	Mohammed & Fox, 1986
Rennetability	Morrissey, 1969	—	Van Hooydonk et al. 1986	Not studied
Acid gelation	Roefs, 1984	Roefs et al. 1990	—	Horne & Parker, 1980
Ethanol stability	Horne & Parker, 1981	De Kruif, 1999	Horne & Parker, 1980	—

Materials and Methods

Milk preparation

Low-heat skim milk powder (NILAC; NIZO food research, Ede, The Netherlands) was reconstituted at 11·25% (w/v) in distilled water; sodium azide (0·2 g/l) was added to prevent microbial growth. Reconstituted skim milk was mixed with distilled water and ethanol to yield 9·00% (w/v) milk solids and 0–20% (v/v) ethanol. The sample containing 0% ethanol will be referred to as control milk hereafter.

Determination of the rennet coagulation time of milk

The rennet coagulation time (RCT) of milk, adjusted to pH 6·6, was determined as described by O'Connell et al. (1998).

For studies on cold renneting, a mixture of milk and rennet (10 μl ml^{-1} milk of a 1:20 aqueous dilution of Maxiren 180, DSM Food Specialties, Delft, The Netherlands) was incubated at 4 °C for 0–240 min; periodically, samples were transferred to a water bath at 30 °C and the time for visible coagulation determined.

Rheolgical properties of rennet-induced gelation of milk

Dynamic oscillatory analysis of rennet-induced coagulation of milk was performed using a Carri-Med Model CSL^2 100 controlled-stress rheometer (TA Instruments, Leatherhead, UK) using Carri-Med software (Version 5.3). The system was fitted with a concentric cylindrical probe, incorporating a recessed acrylic rotor (outer diameter, 23·05 mm; recess, 4·00 mm; cylinder immersed to a depth of 30·00 mm), held in a cylindrical cup (inner diameter, 25·00 mm). Maxiren 180 (75 μl of an aqueous 1:10 dilution) was added to 11 ml milk, which had been tempered at 30 °C for 15 min prior to analysis, and the mixture placed immediately in the measurement cup at 30±0·2 °C. To prevent dehydration, a thin layer of liquid paraffin was poured on top of the samples. The storage modulus, G′, of the sample was recorded continuously at a low amplitude shear strain (0·01 Pa), at a frequency of 1 Hz, over 90 min at 30 °C.

Diffusing wave spectroscopy

Milk was tempered at 30 °C, rennet was added (10 μl ml^{-1} of a 1:20 aqueous mixture of Maxiren 180) and the rennet-induced coagulation at 30 °C was followed by diffusing wave spectroscopy (DWS), as described by Vasbinder et al. (2003). The value of the relaxation time $\tau_{1/2}$, i.e., the time at which the auto-correlation curve had decayed to 50% of its plateau level, reflects the restriction of mobility of the particles in solution. In DWS, a relaxation time is, as in classical dynamic light scattering, directly related to a particle diffusivity and therefore to particle size and interaction *via* the generalized Stokes-Einstein relation. During the initial stages of flocculation, the relaxation time is therefore a direct measure of particle growth (i.e., size). To eliminate the influence of background viscosity, the relaxation time was normalised according to $\tau_{1/2,\,normalised}\,(t)=\tau_{1/2}\,(t)/\tau_{1/2}\,(0)$, where $\tau_{1/2}\,(0)$ is the relaxation time at t=0 min. The lag-time of the rennet-induced coagulation of milk, i.e., the time required for sufficient enzymatic hydrolysis of κ-casein to occur to initiate micellar aggregation, was taken as the time at which $\tau_{1/2,\,normalized}>1{\cdot}0$. The relative aggregation constant of renneted casein micelles was calculated according to von Smoluchowski (1917) kinetics; application of such kinetics to rennet-induced aggregation of casein micelles yields a linear relationship between incubation time and the molecular weight of the aggregates (Payens et al. 1977; Dalgleish et al. 1981). The slope of this function represents the rate constant. Since the relaxation time is directly related to particle size, a relative aggregation constant can be derived from the slope of the linear region of a plot of $(\tau_{1/2})^3$ *versus* incubation time.

Influence of ethanol on the proteolytic activity of chymosin

Size exclusion high-performance liquid chromatography (SE-HPLC) was used to study the hydrolysis of κ-casein by chymosin. Aliquots of milk (2 ml) were incubated with Maxiren (10 μl ml^{-1} of a 1:20 aqueous solution) at 31 °C and, after 0, 15, 30, 60 or 90 min, portions were removed and mixed with 4 ml 12% (w/v) trichloroacetic acid (TCA) to inactivate chymosin and precipitate the caseins and whey proteins. The filtrates, containing glycomacropeptide (GMP) derived from the enzymatic hydrolysis of κ-casein, were analysed by SE-HPLC, as described by Van Hooydonk & Olieman (1982).

Chymosin activity was assayed directly using the synthetic heptapeptide (Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu; Bachem Feinchemikalien AG, Switzerland), as described by Hurley et al. (1999). The substrate solution (1 mg ml^{-1};

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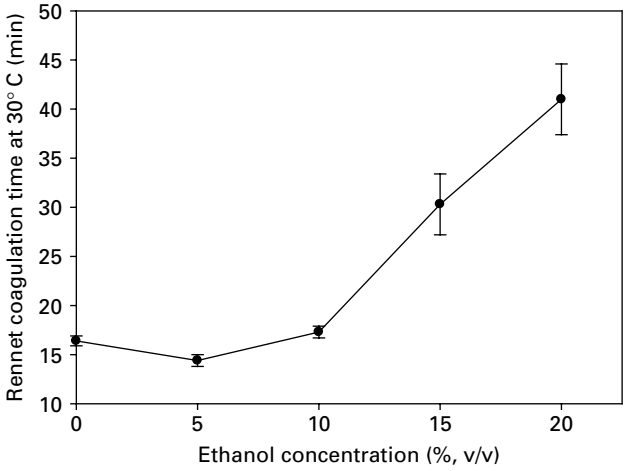

Fig. 1. Influence of ethanol concentration on the rennet coagulation time of milk. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

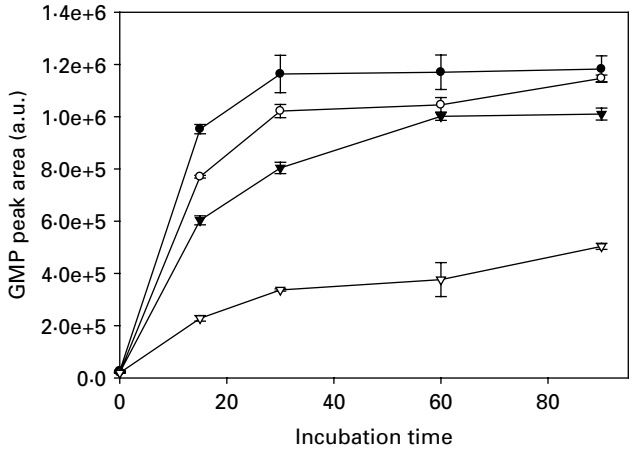

Fig. 2. Influence of renneting time at 30 °C on the level of glycomacropeptide (GMP) produced in milk containing 0 (-●-), 5 (-○-), 10 (-▼-) or 20 (-▽-) % (v/v) ethanol. Values are means of data from triplicate experiments on individual milk samples, with the standard deviation indicated by vertical error bars.

30 µl) was mixed with 200 µl 100 mM-sodium formate buffer, pH 3·2, containing 0, 7·5, 15 or 30% (v/v) ethanol and 0·5 g sodium azide/l. To initiate the reaction, 70 µl of a buffered solution of Maxiren 180 (500 µl l^{-1} in 0·01 M-sodium acetate buffer, pH 5·5) was added and the mixture incubated at 37 °C for 4 h; the reaction was terminated by heating samples at 70 °C for 10 min. Samples were centrifuged at 16 000 ***g*** for 10 min and analysed by RP-HPLC, as described by Hurley et al. (1999).

Results

Influence of ethanol on rennet-induced coagulation of milk

The RCT of milk containing 5% ethanol was lower than that of control milk, whereas that of milk containing 10% ethanol was similar to that of control milk; the RCT of milk containing 15 or 20% ethanol was ~2 or ~3 times higher, respectively, than that of control milk (Fig. 1). Similar effects of ethanol were observed on the RCT of serum protein-free milk (results not shown).

On cold renneting, which allows the primary stage of rennet-induced coagulation to proceed without the subsequent gelation of *para*-casein micelles, the RCT of control milk (i.e., the time of subsequent incubation at 30 °C required to induce visible coagulation) decreased with incubation time to ~12 min after 240 min at 4 °C; RCT decreased more rapidly with incubation time in milk containing 5, 10 or 20% ethanol than in control milk and reached a considerably lower value after 240 min at 4 °C (data not shown). The RCT of cold-renneted milk was also determined at temperatures in the range 4–20 °C. Coagulation of control milk was not observed at a temperature <20 °C, but milk containing 5% ethanol coagulated at 15 °C, albeit slowly. Milk containing 10 or 20% ethanol coagulated at a temperature as low as 4 °C (data not shown). In separate experiments, milk was renneted at 4 °C for 140 min, followed by addition of ethanol to a final concentration of 0–20% and determination of the RCT at 30 °C. Control milk coagulated after 11 min at 30 °C but samples containing 5, 10 or 20% ethanol coagulated after 150, 25 or 10 sec, respectively (data not shown).

Influence of ethanol on the proteolytic activity of chymosin

The rate of rennet-induced production of GMP was highest in control milk and lowest in milk containing 20% ethanol (Fig. 2). After incubation at 31 °C for 90 min, little difference in the amount of GMP was observed between control milk and milk containing 5 or 10% ethanol, but the level of GMP was considerably lower in milk containing 20% ethanol (Fig. 2). Urea-PAGE analysis showed that the extent of chymosin-induced hydrolysis of α_{s1}- and β-caseins in sodium caseinate was also reduced with increasing ethanol content (0–20%; data not shown).

The amount of the tripeptide, [NO_2-Phe]-Arg-Leu, produced from the heptapeptide substrate Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu by chymosin was inversely proportional to the ethanol content of the reaction mixture; the amount of tripeptide produced in a reaction mixture containing 20% ethanol was ~10 times lower than that in an ethanol-free mixture (Fig. 3).

Influence of ethanol on the rennet-induced formation of a milk coagulum

Little difference was observed between the elastic modulus, *G'*, of control milk and milk containing 10% ethanol

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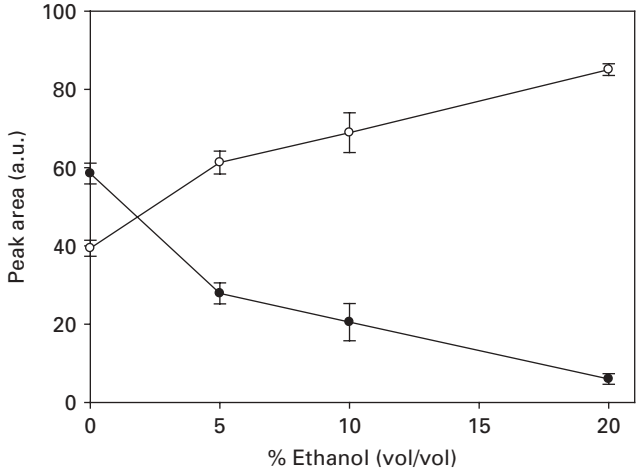

Fig. 3. Influence of ethanol concentration on the hydrolysis of the heptapeptide Pro-Thr-Glu-Phe-[NO_2-Phe]-Arg-Leu (-○-) by chymosin to yield the tripeptide [NO_2-Phe]-Arg-Leu (-●-) in formate buffer (pH 3·2) during incubation at 37 °C for 4 h. Values are means of data from triplicate experiments on individual samples, with the standard deviation indicated by vertical error bars.

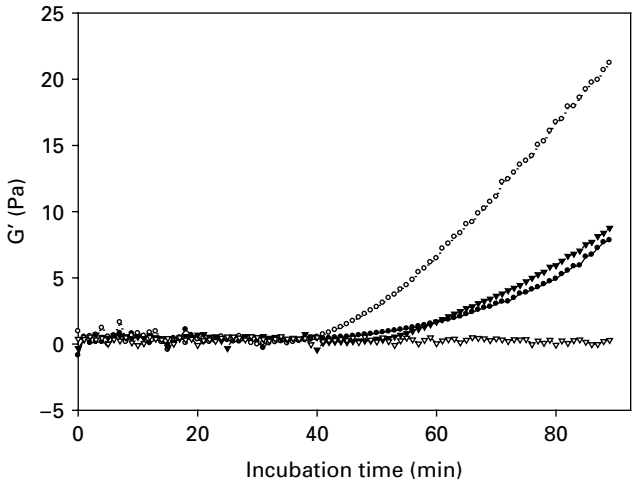

Fig. 4. Influence of incubation time at 30 °C after addition of rennet on the elastic modulus (G′) of milk containing 0 (-●-), 5 (-○-), 10 (-▼-) or 20 (-▽-) %, v/v, ethanol. Values are means of data from triplicate experiments on individual milk samples.

throughout renneting (Fig. 4). In milk containing 5% ethanol, the rate of increase in *G′* and the final value of *G′* after 90 min was considerably higher than that in control milk, whereas no increase in *G′* was observed in milk containing 20% ethanol throughout renneting for 90 min (Fig. 4).

Aggregation of κ-casein-depleted casein micelles in the presence of ethanol was also studied using diffusing wave spectroscopy (DWS; Fig. 5). Compared with control milk, increases in the relaxation time $\tau_{1/2}$, which reflects the extent of restriction of movement of casein micelles, for milk containing 6, 10 or 15% ethanol were observed after a longer incubation time (Fig. 5); the lag time, i.e., the time before an increase in $\tau_{1/2}$ was detected, was ∼16, ∼22, ∼35 or ∼51 min for milk containing 0, 6, 10 or 15% (v/v) ethanol; in milk containing 20% ethanol, no increase in $\tau_{1/2}$ was observed throughout the 80 min of analysis. The rate of increase in $\tau_{1/2}$, after the lag time, was considerably higher in milk containing 6, 10 or 15% ethanol than in control milk (Fig. 5); relative aggregation constants were, according to von Smoluchowski kinetics, estimated to be ∼18, 170, 53 or 32 for milk containing 0, 6, 10 or 15% ethanol.

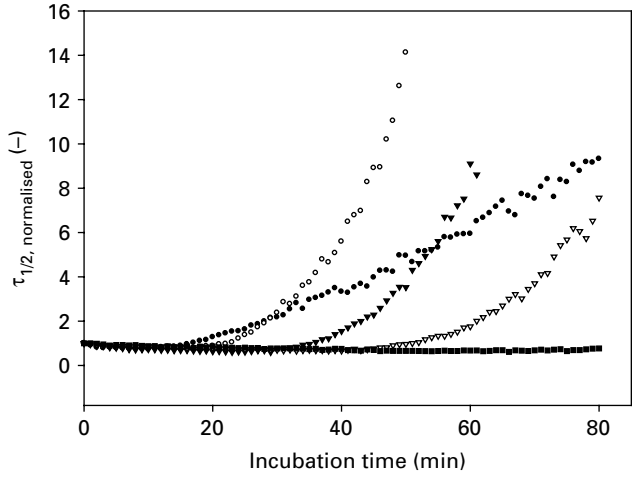

Fig. 5. Influence of incubation time with chymosin at 30 °C on the normalized $\tau_{1/2}$, determined using diffusing wave spectroscopy (DWS) of milk containing 0 (-●-), 6 (-○-), 10 (-▼-), 15 (-▽-) or 20 (-■-) % (v/v) ethanol. Values are means of data from triplicate experiments on individual milk samples.

Discussion

The results presented in this communication suggest a considerable influence of ethanol on the rennet-induced coagulation of milk, which has not been reported previously. As shown in Fig. 1, the RCT of milk was slightly shorter in the presence of 5% ethanol than in control milk, but considerably longer in the presence of 15 or 20% ethanol. Since similar results were observed for whey protein-free milk (data not shown); it is unlikely that the observed effects were due to ethanol-induced changes in the whey proteins. It is more likely that the influence of ethanol on the rennet-induced coagulation of milk is related to ethanol-induced changes in the colloidal stability of the casein micelles or activity of chymosin, as described below.

On addition of ethanol to an aqueous system, e.g., milk, the dielectric constant of the system is reduced (Akerlof, 1932), which induces various changes in the

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casein micelles: (1) the protruding C-terminal region of κ-casein on the micellar surface collapses, which leads to reduced intermicellar steric repulsion, as well as to a reduction in the hydrodynamic radius of the micelles (Horne, 1984; Horne & Davidson, 1986); (2) the pKa of glutamate and aspartate residues is increased, while that of the basic residues, lysine, arginine and histidine, is not affected (Jukes & Schmidt, 1934), which leads to a decrease in the net negative surface charge; (3) the solubility of calcium and phosphate is reduced, leading to an increased level of calcium and phosphate associated with the casein micelles (Pierre, 1985). Ethanol-induced collapse of the κ-casein layer, reduction in micellar charge and precipitation of calcium phosphate are all likely to reduce micellar stability.

The process of rennet coagulation may be divided into two discreet, but partially-overlapping, stages (see Hyslop, 2003): the primary stage of rennet coagulation, which involves the enzymatic hydrolysis of κ-casein by chymosin, and the secondary stage, which involves the calcium-induced aggregation of κ-casein-depleted para-casein micelles, leading ultimately to the formation of a coagulum. The ethanol-induced reduction in the rate of production of GMP (Fig. 2) suggests that the primary stage of rennet coagulation is slowed considerably by ethanol. A similar effect was evident from DWS data (Fig. 5), where the lag-time increased with increasing concentration of ethanol. The reduced rate of the primary stage of rennet-induced coagulation is probably due to an ethanol-induced reduction in chymosin activity, as indicated by a reduced degree of hydrolysis of heptapeptide substrate by chymosin in a given time (Fig. 3). Simon et al. (2001) showed that the activity of trypsin and α-chymotrypsin is reduced considerably in the presence of 20% ethanol, and related this to ethanol-induced conformational changes in the enzyme molecule; ethanol-induced conformational changes may also occur in chymosin. The reduced rate of production of GMP may also be related partially to the ethanol-induced collapse of the protruding C-terminal region of the κ-casein layer on the micellar surface, which may restrict its availability for hydrolyis by chymosin. Thus, it appears clear that ethanol-induced increases in the RCT of milk (Fig. 1), as well as the increased lag-time prior to rennet-induced coagulation of milk, are, at least partially, due to a reduced rate of enzymatic hydrolysis of κ-casein.

Ethanol-induced increases in the rate constant for micellar aggregation derived from data in Fig. 5 indicate that ethanol enhances the secondary stage of rennet-induced coagulation. These observations are supported by the fact that ethanol reduced the coagulation time of milk after cold renneting (data not shown). The ethanol-induced reduction in the level of steric stabilization of casein micelles promotes their susceptibility to aggregation (Horne, 1984). Furthermore, the amount of GMP release from the micelles (Fig. 2) at the time of coagulation (Fig. 1) clearly indicates that in the presence of ethanol, a lower level of κ-casein hydrolysis is required to induce the aggregation of the micelles.

The reduction in gel strength (Fig. 4) and aggregation constant with increasing ethanol content >5% may be related to the fact that addition of ethanol results in the precipitation of soluble calcium onto the micelles (Pierre, 1985). A reduction in the level of soluble calcium in milk reduces the rate of aggregation of *para*-casein micelles (Dalgleish, 1983) and thus, at a given time, the strength of the coagulum formed. Furthermore, from combination of data shown in Figs. 1 and 2, it is apparent that at a higher level of ethanol, more intact κ-casein is present at the micellar surface when coagulation commences; a high level of intact κ-casein on the micellar surface may reduce the strength of the coagulum formed, analogous to effects for acid-induced coagulation of milk reported by Roefs et al. (1990) and Lucey et al. (2000).

At low temperatures, β-casein aids in the stabilisation of the casein micelles, but this effect is absent at a temperature >20 °C (De Kruif & Roefs, 1996); the steric stabilization provided by β-casein is probably sufficient to prevent rennet-induced coagulation of control milk at a temperature <20 °C. The addition of ethanol reduces steric stabilization (Horne, 1984; Horne & Davidson, 1986) and may thus enable rennet-induced coagulation <20 °C. The progressive reduction in minimum coagulation temperature with increasing ethanol content (0–20%; data not shown) is consistent with a progressive reduction in the hydrodynamic radius of the casein micelles with increasing ethanol content (0–20%; Horne, 1984), suggesting lower micellar stability.

In conclusion, it is clear that ethanol affects both the primary (hydrolysis of κ-casein) and secondary (aggregation of destabilised micelles and subsequent gel formation) stages of rennet-induced coagulation of milk. The magnitude of the effect on each stage, and hence the net total effect, depends on the concentration of ethanol. The reduction in the rate of enzymatic hydrolysis of κ-casein was more extensive than was apparent from ethanol-induced increases in RCT, since the aggregation of para-casein micelles and the formation of a subsequent gel are clearly accelerated by the presence of ethanol and a lower level of κ-casein hydrolysis is required for initiation of coagulation in the presence of ethanol.

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References

Akerlof G 1932 Dielectric constants of some organic solvent-water mixtures at various temperatures. *Journal of the American Chemical Society* **54** 4125–4139

Creamer LK, Plowman JE, Liddell MJ, Smith MH & Hill JP 1998 Micelle stability: κ-casein structure and function. *Journal of Dairy Science* **81** 3004–3012

Dalgleish DG, Payens TAJ & Brinkhuis, J 1981 The rate constants for the aggregation of rennet-treated casein micelles. *Netherlands Milk and Dairy Journal* **35** 381–383

Dalgleish DG 1983 Coagulation of rennet bovine casein micelles – dependence on temperature, calcium-ion concentration and ionic strength. *Journal of Dairy Research* **50** 31–340

Dalgleish DG 1992 The enzymatic coagulation of milk. In *Advanced Dairy Chemistry, Volume 1: Proteins*, 2nd Edn. pp. 597–619 (Ed. PF Fox). London: Elsevier Applied Science Publishers

Dalgleish DG 1998 Casein micelles as colloids: Surface structures and stabilities. *Journal of Dairy Science* **81** 3013–3018

De Kruif CG 1999 Casein micelle interactions. *International Dairy Journal* **9** 183–188

De Kruif CG & Holt C 2003 Casein micelle structure, functions and interactions. In *Advanced Dairy Chemistry, Volume 1: Proteins*, 3rd Edn. pp. 233–276 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

De Kruif CG & Roefs SPFM 1996 Skim milk acidification at low temperatures: A model for the stability of casein micelles. *Netherlands Milk and Dairy Journal* **50** 113–120

Fox PF, O'Connor TP, McSweeney PLH, Guinee TP & O'Brien NM 1996 Cheese: physical, biochemical and nutritional aspects. *Advances in Food and Nutritional Research* **39** 163–328

Holt C 1992 Structure and stability of casein micelles. *Advanced Protein Chemistry* **43** 63–151

Holt C & Horne DS 1996 The hairy casein micelle: Evolution of the concept and its implications for dairy technology. *Netherlands Milk and Dairy Journal* **50** 85–111

Horne DS 1984 Steric effects in the coagulation of milk by ethanol. *Biopolymers* **23** 989–993

Horne DS 2003 Ethanol stability. In *Advanced Dairy Chemistry, Volume 1: Proteins* 3rd Edn. pp. 975–999 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

Horne DS & Parker TG 1980 The pH sensitivity of the ethanol stability of individual cow milk. *Netherlands Milk and Dairy Journal* **34** 126–130

Horne DS & Parker TG 1981 Factors affecting the ethanol stability of milk. IV. Effect of forewarming. *Journal of Dairy Research* **35** 405–415

Horne DS & Davidson CM 1986 The effect of environmental conditions on the steric stabilzation of casein micelles. *Colloid and Polymer Science* **264** 727–734

Hurley MJ, O'Driscoll BM, Kelly AL & McSweeney PLH 1999 Novel assay for determination of residual coagulant activity in cheese. *International Dairy Journal* **9** 553–558

Hyslop DB 2003 Enzymatic coagulation of milk. In *Advanced Dairy Chemistry, Volume 1: Proteins*, 3rd Edn. pp. 839–878 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

Jukes TH & Schmidt CLA 1934 The apparent dissociation constants of certain amino acids and related substances in water-ethanol mixtures. *Journal of Biological Chemistry* **105** 359–371

Lucey JA & Singh H 2003 Acid coagulation of milk. In *Advanced Dairy Chemistry, Volume 1: Proteins*, 3rd Edn. pp. 1001–1062 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

Lucey JA, Tamehana M, Singh H & Munro PA 2000 Rheological properties of milk gels formed by a combination of rennet and glucono-δ-lactone. *Journal of Dairy Research* **67** 415–427

Mohammed KS & Fox PF 1996 Heat and ethanol-induced coagulation of milk. *Irish Journal of Food Science and Technology* **10** 47–55

Morrissey PA 1969 The rennet hysteresis of heated milk. *Journal of Dairy Research* **36** 333–341

O'Connell JE & Fox PF 2003 Heat-induced coagulation of milk. In: *Advanced Dairy Chemistry, Volume 1: Proteins*, pp. 879–945 (Eds PF Fox & PLH McSweeney). New York: Kluwer Academic/Plenum Publishers

O'Connell JE, Fox PD, Tan-Kintia R & Fox PF 1998 Effect of extracts from tea, coffee and cocoa on the colloidal stability of milk. *International Dairy Journal* **8** 689–693

Payens TAJ, Wiersma AK & Brinkhuis J 1977 Enzymatic clotting processes. 1. Kinetics of enzyme-triggered coagulation processes. *Biophysical Chemistry* **6** 253–261

Pierre A 1985 Milk coagulation by alcohol – studies on the solubility of the milk calcium and phosphate in alcoholic solutions. *Lait* **65** 201–212

Roefs SPFM, Van Vliet T, Van den Bijgaart HJCM, De Groot-Mostert AEA & Walstra P 1990 Structure of casein gels made by combined acedification and rennet action. *Netherlands Milk and Dairy Journal* **44** 159–188

Rollema HS 1992 Casein association and micelle formation. In *Advanced Dairy Chemistry, Volume 1: Proteins* (Ed. PF Fox). London: Elsevier Applied Science Publishers

Rose D 1961 Variations in the heat stability and composition of milk from individual cows during lactation. *Journal of Dairy Science* **44** 430–441

Simon LM, Kotorman M, Garab G & Laczko I 2001 Structure and activity of trypsin and α-chymotrypsin in aqueous organic media. *Biochemical and Biophysical Research Communications* **280** 1367–1371

Singh H & Creamer LK 1992 Heat stability of milk. In: *Advanced Dairy Chemistry, Volume 1: Proteins*, pp. 621–656 (Ed. PF Fox). London: Elsevier Applied Science Publishers

Van Hooydonk ACM & Olieman C 1982 A rapid and sensitive high-performance liquid-chromatography method of following the action of chymosin in milk. *Netherlands Milk and Dairy Journal* **36** 153–158

Van Hooydonk ACM, Boerrigter IJ & Hagedoorn HG 1986 pH-Induced changes of casein micelles in milk and their effect on renneting. 2. Effect of pH on renneting of milk. *Netherlands Milk and Dairy Journal* **40** 297–313

Vasbinder AJ, Rollema HJ & De Kruif KG 2003 Impaired rennetability of heated milk: study of enzymatic hydrolysis and gelation kinetics. *Journal of Dairy Science* **86** 1548–1555

Von Smoluchowski M 1917 Versuch einer mathematischen Theorie der Koagulationskinetic kolloider Losungen. *Zeitschrift fur Physikalische Chemie* **92** 129–168

Walstra P 1979 Voluminosity of bovine casein micelles and some of its implications. *Journal of Dairy Research* **46** 317–323

Walstra P 1990 On the stability of casein micelles. *Journal of Dairy Science* **73** 1965–1979