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Prediction of clotting time for milk coagulation by mixtures of proteolytic enzymes

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Kinetics of milk coagulation by three proteolytic enzymes, chymosin, bovine pepsin and neutrase, at varying concentrations, were monitored by thrombelastography. Clotting time for milk coagulation by chymosin was linear (r^2 = 0·997) with the reciprocal of enzyme concentration, whereas the best equation for pepsin (r^2 = 0·997) and neutrase (r^2 = 0·999) was the one relating the reciprocal of clotting time to enzyme concentration. When the effect of mixtures of enzymes on milk coagulation was studied, the best-fitting regression was that of the reciprocal of clotting time on concentration of enzymes for both chymosin–pepsin (r^2 = 0·998) and chymosin–neutrase (r^2 = 0·998) mixtures.

INTRODUCTION

The coagulation of milk is the result of two processes: the attack on the K-casein of the casein micelles by the proteolytic enzymes contained in rennet and the clotting of the micelles which have been destabilised by this enzymatic attack. Milk-coagulating enzymes split bovine K-casein at the Phe_{105}–Met_{106} bond. The rate of the enzymic reaction has been shown to increase linearly with the enzyme concentration, in agreement with a first-order mechanism (Castle & Wheelock, 1972; Dalgleish, 1979). The micelles can begin to coagulate once sufficient of their K-casein has been split, as the hydrophilic glycomacropeptide (GMP) diffuses off into the serum and its stabilising influence is lost (Dalgleish, 1987). The aggregation phase occurs by a random, diffusion-controlled Smoluchowski mechanism (Dalgleish *et al.*, 1981; Green, 1984), the rate of micellar aggregation being independent of their size and little affected by doubling rennet concentration (Dalgleish *et al.*, 1981). Intermicellar linkages which appear on electron micrographs during micellar aggregation become stronger with time bringing the micelles into contact and, eventually, micelles fuse together (Green & Morant, 1981; McMahon & Brown, 1984).

An overall kinetic model for milk coagulation which combines the first-order enzymic reaction and the Smoluchowski aggregation reaction has been proposed (Van Hooydonk & Walstra, 1987). It predicts a linear relation between the clotting time and the reciprocal of the enzyme concentration. Experimental evidence obtained using a Formagraph (McMahon & Brown, 1982), a Sommer-Matsen rennet tester device (Carlson *et al.*, 1985; Singh & Creamer, 1990) or a thrombelastograph (Picón *et al.*, 1993) generally agreed with this model. Linear relationships between the reciprocal of clotting time and the enzyme concentration (Kopelman & Cogan, 1976) or between the clotting time and the reciprocal of the square root of enzyme concentration (Hyslop *et al.*, 1979) have also been suggested.

Chymosin (EC 3.4.23.4), an acid protease present in the abomasum of young ruminants, is preferred as a milk coagulant because of its high specific milk-clotting activity. Due to the shortage in the world supply of calf rennet, other proteases have been developed for use as rennet substitutes. These milk coagulants, animal pepsins or fungal enzymes, are generally acid proteases (Green, 1984), although neutral proteinases of bacterial origin have also been investigated (Puhan, 1969; Puhan & Irvine, 1973). Rennet substitutes show ratios of clotting to proteolytic activity which compare unfavourably with that of chymosin (Dalgleish, 1987; Emmons *et al.*, 1990), and cheese made with them may have flavour and body defects due to the excessive proteolysis. The use of mixtures of a highly proteolytic enzyme with a less proteolytic one has been suggested in order to avoid some of the problems associated with rennet substitutes (Green & Stackpoole, 1975).

However, information on kinetics of milk coagulation by mixtures of coagulating enzymes is scarce. In the present work, experimental data on milk coagulation by mixtures of chymosin and bovine pepsin (EC 3.4.23.1) and of chymosin and neutrase (EC 3.4.24.4) were confronted with mathematical models previously

proposed for milk coagulation by a single enzyme, in order to select the best-fitting equation to predict milk clotting time by mixtures of enzymes.

MATERIALS AND METHODS

Materials

Chymosin used was Maxiren (Gist Brocades NV, Delft, The Netherlands), a commercial preparation of chymosin obtained from *Kluyveromyces lactis*, with a declared content of 900 μg chymosin/ml. Pepsin used was a powder preparation of bovine pepsin with a declared activity of 1000 IMCU/g (Chr. Hansen's Lab., Copenhagen, Denmark). Neutrase used was Neutrase L (Novo España S.A., Madrid, Spain), a liquid preparation with a declared activity of 0·5 AU/g. Standard solutions of chymosin and pepsin were prepared by diluting the enzyme in pH 6·5 phosphate buffered saline (PBS) and standard solutions of neutrase by diluting in pH 7·4 PBS. Raw whole milk (Picón *et al.*, 1993) was used for all experiments.

Milk coagulation

Chymosin, pepsin and neutrase solutions (0·50 ml) were added to 50 ml of milk at 34°C and mixed; 0·35 ml of this mixture was transferred to each of the two vats of a Hellige thrombelastograph model D (Hellige GmbH, Freiburg im Breissgau, Germany), and milk coagulation at 34°C followed as previously described (Picón *et al.*, 1993). Clotting time of milk was time in minutes needed by the thrombelastographic curve to reach an amplitude of 1 mm (Picón *et al.*, 1993). Coagulation trials were carried out in duplicate. Data obtained were adjusted by multiple linear regression analysis to different mathematical models by means of the BMDPIR program (Department of Biomathematics, UCLA, Los Angeles, CA).

RESULTS AND DISCUSSION

Milk coagulation by single enzymes

When chymosin was added to milk at final concentrations in the range 0·08–0·24 mg/litre milk, mean clotting times in the range 13·44–31·63 min were recorded (Table 1). Milk coagulation by chymosin fitted well the kinetic model by Van Hooydonk and Walstra (1987), as shown by the high determination coefficient (r^2 = 0·997) found for the regression of time on the reciprocal of chymosin concentration (Table 2). Similar results had been obtained previously at our laboratory (Picón *et al.*, 1993).

Bovine pepsin added to milk at final concentrations in the range 20–60 IMCU/litre milk gave rise to mean clotting times in the range 8·31–34·94 min (Table 1). Clotting time data for pepsin did not fit the kinetic model by Van Hooydonk and Walstra (1987) as successfully as in the case of chymosin, with an r^2 = 0·989. Bovine pepsin produces soluble N at a faster rate than rennet (Fox, 1969; Green, 1972), and has a clotting to proteolytic activity ratio of only 4·3 versus the high ratio of 40·9 found for chymosin (Dalgleish, 1987). Due to its markedly greater general proteolytic activity, pepsin may simultaneously attack casein fractions other than K-casein and give rise to the formation of degradation products other than GMP. Both the enzymic phase and the micellar aggregation phase might be altered by this non-specific proteolytic activity of pepsin, with the subsequent deviation from the kinetic model by Van Hooydonk and Walstra (1987).

Clotting times not linear with the reciprocal of the concentration of various pepsins have been previously reported (Ketting & Pulay, 1970; Brewer *et al.*, 1984). Clotting time increased with decreasing enzyme concentration to a greater extent than expected, and below a certain pepsin concentration milk clotting did not occur (Ketting & Pulay, 1970). A great disadvantage with pepsin is its strong pH dependence, which can give very long clotting times and weak curd if cheesemilk pH is too high (Andrén & Von Reedtz, 1990). The results of the present study agree with these observations. The equation which best fitted the experimental data for milk coagulation by pepsin, in the present study (Table 1), was the regression of the reciprocal of clotting time on

Table 1. Clotting time[a] for milk coagulation by chymosin, pepsin or neutrase at varying concentrations

Chymosin[b]	Time	Pepsin[c]	Time	Neutrase[d]	Time
0·08	31·63	20	34·94	0·010	47·25
0·12	22·13	30	18·94	0·015	25·38
0·16	17·25	40	13·94	0·020	17·81
0·20	14·31	50	10·13	0·025	13·19
0·24	13·44	60	8·31	0·030	10·75

[a]Minutes to reach an amplitude of 1 mm (mean of two trials).
[b]Chymosin concentration is expressed in mg/litre milk.
[c]Pepsin concentration is expressed in IMCU/litre milk.
[d]Neutrase concentration is expressed in AU/litre milk.

Table 2. Regression equations of clotting time[a] (t) on enzyme concentration for milk coagulation by chymosin (C), pepsin (P) or neutrase (N) at varying concentrations

Enzyme	Equation	r^2
Chymosin[b]	$t = 3{\cdot}5554 + 2{\cdot}2357\ C^{-1}$	0·997
	$t = -13{\cdot}2010 + 12{\cdot}4871\ C^{-1/2}$	0·987
	$t^1 = 0{\cdot}01161 + 0{\cdot}2761\ C$	0·976
Pepsin[c]	$t = -5{\cdot}9408 + 799{\cdot}6835\ P^{-1}$	0·989
	$t = -29{\cdot}6486 + 280{\cdot}8972\ P^{-1/2}$	0·969
	$t^{-1} = -0{\cdot}01726 + 0{\cdot}00229\ P$	0·997
Neutrase[d]	$t = -90213 + 0{\cdot}5499\ N^{-1}$	0·989
	$t = -41{\cdot}6815 + 8{\cdot}6458\ N^{-1/2}$	0·968
	$t^{-1} = -0{\cdot}01491 + 3{\cdot}6010\ N$	0·999

[a]Minutes to reach an amplitude of 1 mm.
[b]Chymosin concentration is expressed in mg/litre milk.
[c]Pepsin concentration is expressed in IMCU/litre milk.
[d]Neutrase concentration is expressed in AU/litre milk.

pepsin concentration (Kopelman & Cogan, 1976), with an $r^2 = 0.997$ (Table 2). If in the equation $t^{-1} = a + b\,P$ (where t is clotting time and P is pepsin concentration), P takes the value $-a/b$, clotting time tends toward infinity and milk does not coagulate. Concentrations higher than $-a/b$ are needed for clotting of milk by pepsin. In the conditions of the present study, a theoretical minimum concentration of 7·6 IMCU/litre may be calculated from the equation for the regression of the reciprocal of clotting time on pepsin concentration (Table 2). A model by Hyslop *et al.* (1979) compared unfavourably to the models mentioned above, with an r^2 of only 0·969 (Table 2), in spite of being based on experiments with pepsin. When the experiment was repeated (data not shown), consistently similar r^2 values were obtained.

Similar results were obtained for milk coagulation by neutrase at concentrations in the range 0·010–0·030 AU/litre, with mean clotting times of 10·75–47·25 min (Table 1). Models by Van Hooydonk and Walstra (1987) and Hyslop *et al.* (1979) resulted in r^2 values of 0·989 and 0·968, respectively (Table 2). Neutrase is able to extensively degrade both α-casein and β-casein (Puhan, 1969), a fact which might considerably perturb micellar aggregation. However, an r^2 value of 0·999 was achieved for the regression of the reciprocal of clotting time on neutrase concentration (Table 2). In the present study, a minimum neutrase concentration of 0·0042 AU/litre for milk coagulation to occur may be calculated from the equation for milk coagulation by neutrase (Table 2). As for pepsin, when the experiment was repeated (data not shown) consistently similar r^2 values were obtained.

Milk coagulation by mixtures of enzymes

No kinetic model for milk coagulation by mixtures of enzymes has been proposed. Ketting and Pulay (1970) concluded that it was not correct to assume a linear relationship between clotting time and enzyme concentration for milk coagulation by chymosin–pepsin mixtures, but did not suggest an alternative equation. *Mucor pusillus* proteinase and calf rennet in a mixture acted synergically rather than additively, clotting activity being more stimulated by trivalent than by divalent cations (Trop & Pinsky, 1971). However, Green and Stackpoole (1975) concluded that activities of *Mucor pusillus* proteinase and swine pepsin were additive, and that mixtures of these two coagulants could easily be prepared and used in cheesemaking. Conversely, Jackman *et al.* (1985) observed that the concerted action of chymosin and a protease from *Pseudomonas fluorescens* T16 on milk coagulation was not additive. The clotting time was higher than expected when T16 protease was mixed with increasing chymosin concentrations. A linear relationship between per cent pepsin in a chymosin–pepsin mixture and k_{20} (minutes from clotting time until a width of 20 mm was reached) was reported (McMahon & Brown, 1985), but the authors did not investigate the effect of proportions in the chymosin–pepsin mixture on clotting time.

Table 3. Clotting time[a] for milk coagulation by mixtures of chymosin and pepsin at varying concentrations

	Chymosin[b]			
Pepsin[c]	0·04	0·08	0·16	0·24
6	50·50	31·06	17·75	12·50
12	39·88	27·00	16·31	11·56
18	32·75	23·63	15·06	10·94
24	28·31	20·94	13·88	10·38

[a]Minutes to reach an amplitude of 1 mm (mean of two trials).
[b]Chymosin concentration is expressed in mg/litre milk.
[c]Pepsin concentration is expressed in IMCU/litre milk.

In the present work, mean clotting times in the range 10·38–50·50 min were obtained for chymosin final concentrations of 0·04–0·24 mg/litre and pepsin final concentrations of 6–24 IMCU/litre (Table 3). When the same chymosin concentrations were combined with neutrase concentrations in the range 0·005–0·030 AU/litre, mean clotting times of 6·63–43·38 min were recorded (Table 4).

Clotting time data from milk coagulation by chymosin–pepsin and chymosin–neutrase mixtures were adjusted to the three regression equations mentioned above (Table 5). The regression of clotting time on the reciprocal of enzyme concentration exhibited the lowest determination coefficients for both chymosin–pepsin ($r^2 = 0.884$) and chymosin–neutrase ($r^2 = 0.795$) mixtures. The non-specific proteolytic activities of pepsin and neutrase may influence both phases, K-casein hydrolysis and micellar aggregation, as mentioned above for the experiment for pure enzyme solutions. The competition between chymosin and pepsin or neutrase for the Phe_{105}–Met_{106} bond, a factor which appears in milk coagulation by mixtures of enzymes, may be partly responsible for the observed large deviation from the linear model by Van Hooydonk and Walstra (1987).

Determination coefficients obtained from the model by Hyslop *et al.* (1979) were 0·925 for milk coagulation by chymosin–pepsin mixtures and 0·818 for milk coagulation by chymosin–neutrase mixtures (Table 5). The regression of the reciprocal of clotting time on enzyme concentration achieved the best-fitting equation for milk coagulation by both chymosin–pepsin ($r^2 = 0.998$) and chymosin–neutrase ($r^2 = 0.998$) mixtures (Table 5).

Table 4. Clotting time[a] for milk coagulation by mixtures of chymosin and neutrase at varying concentrations

	Chymosin[b]			
Neutrase[c]	0·04	0·08	0·16	0·24
0·005	43·38	27·13	15·44	10·81
0·010	29·75	20·75	12·63	9·63
0·020	18·00	14·38	9·94	7·88
0·030	12·25	10·50	8·13	6·63

[a]Minutes to an amplitude of 1 mm (mean of two trials).
[b]Chymosin concentration is expressed in mg/litre milk.
[c]Neutrase concentration is expressed in AU/litre milk.

Table 5. Regression equations of clotting time[a] (t) on enzyme concentration for milk coagulation by mixtures of chymosin (C) and pepsin (P) or chymosin and neutrase (N) at varying concentrations

Enzymes	Equation	r^2
Chymosin,[b] pepsin[c]	$t = -1{\cdot}415\,6 + 1{\cdot}368\,3\ C^{-1} + 71{\cdot}997\,7\ P^{-1}$	0·884
	$t = -19{\cdot}660\,3 + 8{\cdot}974\,0\ C^{-1/2} + 45{\cdot}703\,9\ P^{-1/2}$	0·925
	$t^{-1} = 0{\cdot}002\,3 + 0{\cdot}304\,4\ C + 0{\cdot}000\,88\ P$	0·998
Chymosin, neutrase[d]	$t = -1{\cdot}688\,2 + 0{\cdot}802\,8\ C^{-1} + 0{\cdot}085\,0\ N^{-1}$	0·795
	$t = -19{\cdot}062\,0 + 5{\cdot}803\,0\ C^{-1/2} + 1{\cdot}748\,2\ N^{-1/2}$	0·818
	$t^{-1} = -0{\cdot}003\,2 + 0{\cdot}352\,4\ C + 2{\cdot}321\,2\ N$	0·998

[a]Minutes to reach an amplitude of 1 mm.
[b]Chymosin concentration is expressed in mg/litre milk.
[c]Pepsin concentration is expressed in IMCU/litre milk.
[d]Neutrase concentration is expressed in AU/litre milk.

It may be concluded, from the results obtained in the present work, that clotting time for milk coagulation by mixtures of enzymes is not always linear with the reciprocal of enzyme concentration. On the contrary, the regression equation of the reciprocal of clotting time on the concentration of enzymes successfully fits kinetics of milk coagulation by mixtures of enzymes, especially for proteases such as pepsin and neutrase which, when used as single coagulants, themselves follow this pattern.

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REFERENCES

Andrén, A. & Von Reedtz, C. (1990). Effects of chromatographically pure bovine chymosin and pepsin A on cheese curd firmness. *J. Dairy Res.*, **57**, 109–17.

Brewer, P., Helbig, N. & Haard, N. F. (1984). Atlantic cod pepsin-characterisation and use as a rennet substitute. *Can. Inst. Food Sci. Technol. J.*, **17**, 38–43.

Carlson, A., Hill, C. G., Jr & Olson, N. F. (1985). Improved assay procedure for determination of milk-clotting enzymes. *J. Dairy Sci.*, **68**, 290–9.

Castle, A. V. & Wheelock, J. V. (1972). Effect of varying enzyme concentration on the action of rennin on whole milk. *J. Dairy Res.*, **39**, 15-22.

Dalgleish, D. G. (1979). Proteolysis and aggregation of casein micelles treated with immobilized or soluble chymosin. *J. Dairy Res.*, **46**, 653–61.

Dalgleish, D. G. (1987). The enzymatic coagulation of milk. In *Cheese: Chemistry, Physics and Microbiology*, Vol. I, ed. P. F. Fox. Elsevier Applied Science, London, pp. 63–96.

Dalgleish, D. G., Brinkhuis, J. & Payens, T. A. J. (1981). The coagulation of differently sized casein micelles by rennet. *Eur. J. Biochem.*, **119**, 257–61.

Emmons, D. B., Beckett, D. C. & Binns, M. (1990). Milk-clotting enzymes. 1. Proteolysis during cheese making in relation to estimated losses of yield. *J. Dairy Sci.*, **73**, 2007–15.

Fox, P. F. (1969). Milk-clotting and proteolytic activities of rennet, and of bovine pepsin and porcine pepsin. *J. Dairy Res.*, **36**, 427–33.

Green, M. L. (1972). Assessment of swine, bovine and chicken pepsins as rennet substitutes for Cheddar cheese-making. *J. Dairy Res.*, **39**, 261–73.

Green, M. L. (1984). Milk coagulation and the development of cheese texture. In *Advances in the Microbiology and Biochemistry of Cheese and Fermented Milk*, eds F. L. Davies & B. A. Law. Elsevier Applied Science Publishers, London, pp. 1–33.

Green, M. L. & Morant, S. V. (1981). Mechanism of aggregation of casein micelles in rennet-treated milk. *J. Dairy. Res.*, **48**, 57–63.

Green, M. L. & Stackpoole, A. (1975). The preparation and assessment of a suitable *Mucor pusillus* Lindt proteinase–swine pepsin mixture for Cheddar cheese-making. *J. Dairy Res.*, **42**, 297–312.

Hyslop, D. B., Richardson, T. & Ryan, D. S. (1979). Kinetics of pepsin-initiated coagulation of K-casein. *Biochim. Biophys. Acta*, **556**, 390–6.

Jackman, D. M., Patel, T. R. & Haard, N. F. (1985). Effect of heat-stable proteases on the kinetic parameters of milk clotting by chymosin. *J. Food Sci.*, **50**, 602–4, 609.

Ketting, F. & Pulay, G. (1970). The properties of rennin–pepsin mixtures. *Proceedings XVIII Int. Dairy Congr.*, International Dairy Federation, Brussels, p. 291.

Kopelman, I. J. & Cogan, U. (1976). Determination of clotting power of milk clotting enzymes. *J. Dairy Sci.*, **59**, 196–9.

McMahon, D. J. & Brown, R. J. (1982). Evaluation of Formagraph for comparing rennet solutions. *J. Dairy Sci.*, **65**, 1639–42.

McMahon, D. J. & Brown, R. J. (1984). Enzymic coagulation of casein micelles: a review. *J. Dairy Sci.*, **67**, 919–29.

McMahon, D. J. & Brown, R. J. (1985). Effects of enzyme type on milk coagulation. *J. Dairy Sci.*, **68**, 628–32.

Picón, A., Gaya, P. & Nuñez, M. (1993). Quantitative determination of chymosin activity by thrombelastography. *Food Chem.*, **47**, 209–12.

Puhan, Z. (1969). Protease composition of a rennet substitute from *Bacillus subtilis* and properties of its component proteases. *J. Dairy Sci.*, **52**, 1372–8.

Puhan, Z. & Irvine, D. M. (1973). Proteolysis by proteases of *Bacillus subtilis* used to make Canadian Cheddar cheese. *J. Dairy Sci.*, **56**, 317–22.

Singh, H. & Creamer, L. K. (1990). A sensitive quantitative assay for milk coagulants in cheese and whey products. *J. Dairy Sci.*, **73**, 1158–65.

Trop, M. & Pinsky, A. (1971). *Mucor pusillus* rennin as a synergist to calf rennet. *J. Dairy Sci.*, **54**, 5–7.

Van Hooydonk, A. C. M. & Walstra, P. (1987). Interpretation of the kinetics of the renneting reaction in milk. *Neth. Milk Dairy J.*, **41**, 19–47.