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KINETIC STUDIES ON THE ACTION OF *MUCOR PUSILLUS, MUCOR MIEHEI* ACID PROTEASES AND CHYMOSINS A AND B ON A SYNTHETIC CHROMOPHORIC HEXAPEPTIDE

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Summary

The action of two milk-clotting fungal proteases from *Mucor pusillus* and *Mucor miehei* and of chymosins A and B on the hexapeptide, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, and on κ-casein were studied.

The effects of pH and temperature on the initial rates of hydrolysis of the hexapeptide were examined. Crystalline chymosin and *M. pusillus* protease exhibited optimal activities around 49 and 55°C, respectively, whereas the optimum temperature for *M. miehei* protease is higher than 63°C. The optimum pH was about 4.7 for both fungal proteases whereas chymosin A and chymosin B exhibited optimal activities around 4.2 and 3.7, respectively. Kinetic parameters were then determined under optimal conditions and/or at pH 4.7. Fungal proteases had k_{cat}/K_m ratios that were similar to each other and that were significantly greater than the ratios obtained for the chymosins. Nervertheless, chymosins had much greater clotting activities towards κ-casein relative to their proteolytic activities towards the synthetic peptide.

Introduction

The action of chymosin (EC 3.4.23.4) on κ-casein has been intensively studied [1]. The preliminary determination of κ-casein primary structure [2] and elucidation of Phe_{105}-Met_{106} as a chymosin-labile peptide bond in κ-casein [3] allowed Hill [4] to undertake the synthesis of several chymosin-sensitive peptides. However, the synthetic peptide (Ser-Leu-Phe-Met-Ala-OMe) that was

most sensitive to chymosin was still a poor substrate relative to κ-casein. Later, the exact sequence of bovine κ-casein around the Phe-Met bond was determined [5—7] to be Leu_{103}-Ser-Phe_{105}-Met_{106}-Ala-Ile_{108}. Based on these results, a series of peptide substrates for chymosin were synthesized [8,9]. One of them, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe was proposed by Raymond et al. [10] as a standard for chymosin and bovine pepsin A activity determination; it was also a good substrate for porcine pepsin [11].

Various animal, plant and microbial proteases have been suggested as milk coagulants [12]. Of the multitude of bacterial and fungal substitutes for animal proteases proposed, only those from *Bacillus subtilis*, *Bacillus cereus* [13], *Mucor pusillus* Lindt [14], *Mucor miehei* [15,16] and *Endothia parasitica* [17] are of interest for cheese-making and only the fungal proteases are intensively used by the cheese industry. The molecular and enzymatic characteristics of the acid proteases from *M. pusillus* Lindt and *M. miehei* are the closest to those of chymosin.

In order to test the hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe as a standard substrate for the assay of *M. pusillus* and *M. miehei* proteases, and to compare these proteases with chymosin, we have undertaken kinetic studies on the action of both fungal and chymosin enzymes on this peptide. Since most of the studies on chymosin have been carried out with the crystalline enzyme which contains at least three components, chymosins A, B and C [18—20], and since little information is available on the enzymatic properties of each of the two main components, A and B, we have extended our study to include both chymosins A and B.

We also investigated whether there is a correlation between the proteolytic activities of these enzymes toward the standard hexapeptide and their abilities to hydrolyze and trigger the clotting process of bovine κ-casein. Since data are lacking on the action of bovine pepsin A on κ-casein, we also determined the clotting activity of this enzyme.

Materials and methods

Enzymes

Mucor miehei NRRL 3420 and *Mucor pusillus* Lindt proteases were gifts from Dr. Sternberg (Miles Laboratory Inc., Marschall Division, Elkhart, IN, U.S.A.) and from Société Vitex France (Meito, Sangyo for Europe), respectively. The homogeneity of each fungal protease preparation was checked by polyacrylamide gel electrophoresis by the method of Davis [21].

Chymosin was purified and crystallized from commercial rennet powder (Chr. Hansen's Laboratory Ltd.) as described by Castle and Wheelock [22]. Subsequently, crystalline chymosin was fractionated on DEAE-cellulose column (Whatman DE 32) according to the method of Foltmann [20]. Chymosins A and B thus obtained were chromatographed again under the same conditions. Fractions corresponding to chymosin A and chymosin B were dialyzed against distilled water and freeze-dried. Crystalline chymosin and chymosins A and B are indistinguishable by Davis' electrophoresis method [21]. However, our preparations of chymosins were considered to be pure since their specific activ-

ities (clotting activity on κ-casein/absorbance at 280 nm) were constant along the elution peaks.

Bovine pepsin A was purified from commercial powder (Chr. Hansen's Laboratory Ltd.) by a three step procedure: DEAE-cellulose (Whatman, DE 32) chromatography according to Garnot et al. [23] and two successive Sephadex G-75 (Pharmacia) gel filtrations (Column 3 × 180 cm, flow rate 25 ml/h) in 0.05 M trisodium citrate/0.1 M NaCl buffer (pH 5.8). Such a preparation is heterogeneous. At least three components, which appear to differ in their organic phosphate content, can be obtained by chromatography on hydroxyapatite [24]. The enzymatic activities of these different components on the hexapeptide are presently being investigated.

The proteases were dissolved in 0.05 M sodium phosphate buffer (pH 6.0). The enzyme concentrations were determined spectrophotometrically using the following absorption coefficients, and taking into account the scattered light: $\epsilon_{1cm}^{1mg/ml} = 1.53$ at 277.5 nm for crystalline chymosin [10] and for chymosins A and B; $\epsilon_{1cm}^{1mg/ml} = 1.48$ at 280 nm for bovine pepsin A [24]; $\epsilon_{1cm}^{1mg/ml} = 1.58$ at 278 nm for *M. pusillus* protease; and $\epsilon_{1cm}^{1mg/ml} = 1.36$ at 278 nm for *M. miehei* protease.

The absorption coefficients of the fungal proteases were determined by us from amino acid analyses and spectrophotometric studies. Molecular weights were assumed to be 35 650 [25], 33 367 [24], 32 500 [26] and 34 000 [16] for chymosins, bovine pepsin A, *M. pusillus* protease and *M. miehei* protease, respectively. The enzyme solutions were divided into 100-μl fractions and kept frozen until used.

Peptide

The hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (M_w = 836 in trifluoroacetate salt form) was synthesized by the Merrifield solid-phase method [9] or purchased from Bachem., CA, USA. A 0.7 mM peptide stock solution was prepared by stirring the crystallized peptide in 0.1 M sodium acetate buffer (pH 4.7) for 16 h at 4°C. This solution was filtered through a 0.45 μm Millipore filter and the exact concentration was determined spectrophotometrically using the absorption coefficient $\epsilon_{max} = 8300 \pm 100\ M^{-1} \cdot cm^{-1}$ at 279.5 nm [10].

Kinetic studies with the synthetic hexapeptide

It was shown by thin-layer chromatography and N-terminal group analysis using leucine aminopeptidase [27] and dansyl chloride [28] that only the Phe(NO_2)-Nle bond in the hexapeptide is split by *M. miehei* protease, *M. pusillus* protease and chymosins A and B, as previously demonstrated for crystalline chymosin [9] and bovine pepsin A [11].

Enzymic cleavage of the Phe(NO_2)-Nle bond of the synthetic hexapeptide was followed by difference spectrophotometry [29] using a Beckman Acta M VI spectrophotometer equipped with an automatic sampling system. The absorbance change at 310 nm was monitored for 5 min during which less that 10% of the substrate was hydrolysed. Except for the determination of the temperature optimum, reactions were carried out at 30 ± 0.1°C. The ultraviolet spectrum of the hexapeptide exhibits an absorption maximum at 279.5 nm in the pH range 1.5—12. The spectrum of the reaction product Leu-Ser-Phe(NO_2)

undergoes a bathochromic shift from 279.5 to 283.5 nm between pH 1.5 and 5.5. All the previous studies were done at pH 4.7 [9,10,30—32]. At this pH, the difference spectrum (cleavage product vs. substrate) showed a maximal change in absorbance at 310 nm [9] with a $\Delta\epsilon = 1000 \pm 100\ M^{-1} \cdot cm^{-1}$ [30].

Determination of the pH optimum. The initial rate of hydrolysis of the hexapeptide was determined in quadruplicate at different pH values between 2.7 and 5.6. Corrections were made for the variations of $\Delta\epsilon$ values as a function of pH [30]. The pH of the peptide solution was adjusted by adding 50 µl NaOH or HCl of the appropriate molarity to 1 ml 0.1 mM peptide solution in 0.1 M sodium acetate buffer, pH 4.7. The pH at the end of the reaction was unchanged.

Influence of temperature on the initial rate of hydrolysis. Initial rates of cleavage were monitored in duplicate at different temperatures in the range 25—63°C. $\Delta\epsilon$ was unaffected by temperature.

Evaluation of kinetic parameters. To 0.1—0.62 mM substrate in 1 ml, 15 µl of enzyme solution was added.

k_{cat} and K_m were estimated from experiments carried out with 5—12 substrate concentrations. Slope and intercept from plots of $1/v$ vs. $1/s$ [33] and v vs. v/s [34] were determined by unweighted linear regression analysis. The precision (95% confidence limits) of the kinetic parameters was estimated.

Determination of proteolytic activity. The previously described method [10] was slightly modified. To 2.5 ml of 0.2 mM substrate solution, 50 µl of enzyme solution was added. To ensure accurate assays the initial slope of the absorbance change with time was plotted against seven different enzyme concentrations in triplicate. Proteolytic activity was determined at four different pH values for crystalline chymosin and chymosins A and B.

Determination of clotting activity. Clotting tests on κ-casein were performed by the method of Douillard and Ribadeau Dumas [35] in duplicate, with 8 different enzyme concentrations, with a 0.2% κ-casein solution in 0.05 M citrate/0.075 M NaCl buffer (pH 5.3) at 30°C. κ-Casein was prepared according to Zittle and Custer [36].

Results

Kinetic studies with the synthetic peptide

The effects of pH and temperature on the initial rates of reaction were determined at a single substrate concentration. Initial velocity vs. temperature plots obtained with crystalline chymosin, or with *M. pusillus* and *M. miehei* proteases are shown in Fig. 1. Under the conditions tested, the temperature optimum was not reached for *M. miehei* protease, whereas crystalline chymosin and *M. pusillus* protease exhibited activity optima at approx. 49 and 55°C, respectively. Plots for initial velocity vs. pH were made for chymosins A and B and for the fungal proteases (Fig. 2). Chymosins A and B hydrolysed the synthetic peptide with velocity maxima at approx. pH 4.2 and 3.7, respectively. *M. pusillus* and *M. miehei* proteases had a pH optimum nearby 4.7. However, both fungal proteases may have another pH optimum between 3.7 and 3.8 since we always observed a shoulder around this pH in four determinations performed independently. This could also indicate that there was some hetero-

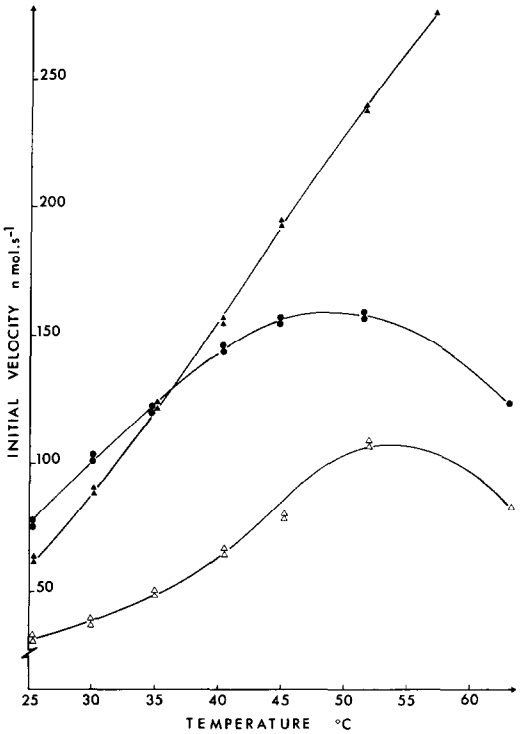

Fig. 1. Effect of temperature on the initial velocity of hydrolysis of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe by crystalline chymosin (●), *M. miehei* protease (▲), and *M. pusillus* protease (△). Substrate concentration 0.2 mM. Final enzyme concentrations were 29.4, 19.8 and 9.0 nM for chymosin, *M. miehei* and *M. pusillus* proteases, respectively.

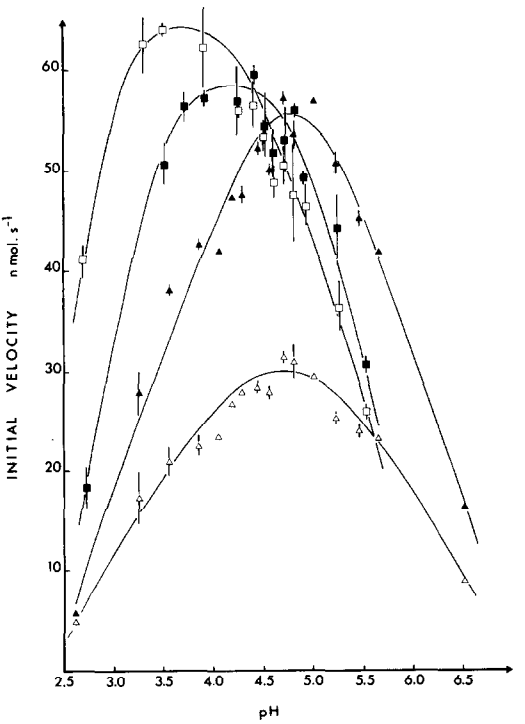

Fig. 2. Effect of pH on the initial velocity of hydrolysis of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe by chymosin A (■), chymosin B (□), *M. miehei* protease (▲) and *M. pusillus* protease (△). Substrate concentration 0.1 mM. Final enzyme concentrations were 28.1, 27.3, 21.5 and 9.8 nM for chymosin A, chymosin B, *M. miehei* protease and *M. pusillus* protease, respectively.

geneity in these enzymic preparations which was not detected under our electrophoretic conditions.

Table I gives the kinetic parameters (k_{cat}, K_m and k_{cat}/K_m) for *M. pusillus* and *M. miehei* proteases and chymosins A and B at pH 4.7, as well as the same kinetic parameters for chymosins A and B at their own pH optima (i.e. 4.2 and 3.7, respectively).

Though the values of k_{cat}/K_m in the two experiments performed independently with *M. pusillus* protease differ appreciably, the kinetic parameters for the fungal acid proteases seem to be remarkably similar. The low solubility and the strong absorbance of the substrate made it impossible to use higher concentrations of substrate. Thus, we had not the best conditions to estimate with the required accuracy the kinetic parameters k_{cat} and K_m for chymosins A and B. Therefore, the most significant parameter for these enzymes is the ratio k_{cat}/K_m. This makes it difficult to compare the chymosins with each other or with the fungal proteases with respect to their k_{cat} and K_m values. Nevertheless, there was no significant difference between the kinetic parameters for chy-

TABLE I

KINETIC PARAMETERS OF CLEAVAGE OF Leu-Ser-Phe(NO_2)$\downarrow$Nle-Ala-Leu-OMe

All experiments were carried out at 30°C in 0.1 M sodium acetate buffer (pH 3.7, 4.2, 4.7). All k_{cat} and K_m values are given with their 0.95 confidence limits. Data for chymosin A and B were obtained with a commercial peptide (Bachem). n = number of initial velocity determinations over concentration range. L-B, Lineweaver-Burk plots; E, Eadie plots.

	pH	Initial substrate concentration (mM)	Final enzyme concentration (nM)	n	Plot	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
M. miehei protease	4.7	0.10—0.28	52.1	10	L-B	0.129 ± 0.022	5.2 ± 0.7	40.3
					E	0.123 ± 0.045	5.1 ± 0.8	41.5
		0.10—0.40	52.9	14	L-B	0.134 ± 0.066	5.6 ± 0.2	41.8
					E	0.135 ± 0.009	5.6 ± 0.1	41.5
M. pusillus protease	4.7	0.10—0.36	47.3	10	L-B	0.092 ± 0.025	2.8 ± 0.4	30.4
					E	0.089 ± 0.053	2.7 ± 0.6	30.3
		0.10—0.28	47.3	10	L-B	0.077 ± 0.017	4.0 ± 0.4	51.9
					E	0.074 ± 0.026	3.9 ± 0.4	52.7
Chymosin A	4.2	0.07—0.59	7.5	18	L-B	0.895 ± 0.07	26.3 ± 0.6	29.4
					E	0.93 ± 0.04	27.3 ± 0.3	29.4
	4.7	0.08—0.50	14.4	39	L-B	1.09 ± 0.13	27.0 ± 1.2	24.8
					E	0.96 ± 0.17	24.4 ± 3.6	25.4
Chymosin B	4.7	0.08—0.50	11.9	39	L-B	1.01 ± 0.09	25.0 ± 1.7	24.8
					E	0.87 ± 0.12	22.2 ± 3.1	25.5
	3.7	0.08—0.62	6.7	22	L-B	0.922 ± 0.11	28.3 ± 0.7	30.7
					E	0.877 ± 0.09	27.3 ± 0.4	31.1
Crystalline chymosin						0.7 [a]	12.3 [a]	17.6 [a]
						0.5 [b]	12.0 [b]	24.2 [b]
Porcin pepsin [a]						0.02	100.0	5000
Bovine pepsin [a]						0.03	54.0	1800

[a] Data from Raymond [11].
[b] Data from Visser et al. [31].

TABLE II

PROTEOLYTIC AND CLOTTING ACTIVITIES

All experiments were carried out at 30°C in 0.1 M sodium acetate buffer (pH 4.7, 4.2, 4.1, 3.7) for proteolytic activity and in 0.05 M sodium citrate/0.075 M NaCl buffer (pH 5.3) for clotting activity. Proteolytic activity was assayed as hydrolyzed peptide (μmol per mg enzyme per s) as described in Materials and Methods. Clotting activity is reciprocal of the amount of enzyme (μg) which gives a clotting time of 100 s at 30°C with 0.2% κ-casein solution. Proteolytic and clotting activities were estimated by linear regression analysis, on the basis of seven (in triplicate) and eight (in duplicate) different enzyme concentrations. Numbers in parentheses indicate the 0.95 confidence limits.

	Proteolytic activity ($\mu mol \cdot mg^{-1} \cdot s^{-1}$)				Clotting activity (μg^{-1})	Clotting activity/proteolytic activity (pH 4.7) × 10^3
	pH 4.7	pH 4.2	pH 4.1	pH 3.7		
M. Miehei protease	139 (±5)				0.4 (±0.05)	2.9
M. pusillus protease	137 (±4)				0.7 (±0.06)	5.1
Crystalline chymosin	93 (±4)		112 (±6)		2.5 (±0.1)	26.9
Chymosin A	95 (±5)	128 (±3)	114 (±4)		2.3 (±0.08)	24.2
Chymosin B	93 (±7)		124 (±3)	149 (±4)	2.1 (±0.1)	22.3
Bovine pepsin A	2540 (±40)				2.6 (±0.17)	1.0

mosins A and B at pH 4.7 or at their own pH optima. Also, as seen in Table I, the K_m values are appreciably lower (10-fold) for the fungal proteases than for the other enzymes. In contrast, the k_{cat} values are 5—8-fold higher with chymosins A and B than for the fungal enzymes.

Proteolytic and clotting activities

Table II summarizes the activities of the different proteases on κ-casein and on the hexapeptide.

Both chymosins exhibited the same proteolytic activity at pH 4.7, but they had only about 70% of the proteolytic activity of the fungal proteases. Bovine pepsin A was a far more efficient enzyme than the chymosins or *M. pusillus* and *M. miehei* proteases towards the synthetic substrate, though its pH optimum is certainly lower [11]. At their own pH optima, chymosins A and B significantly differed in their proteolytic activities.

Bovine pepsin A and chymosins showed very similar clotting activities towards κ-casein at pH 5.3. The latter activities were 3.5—6-fold greater than those obtained with the fungal proteases.

Discussion

Milk clotting enzymes have usually been assayed by measuring the time needed for the enzyme to result in milk clotting. However, since many factors influence the clot formation, standardized conditions must be carefully followed to obtain reliable and reproducible results. Nevertheless, even under the best standardized conditions most of the readily-employed methods [35,37] rely on a substrate (κ-casein, reconstituted skimmed milk) which is subject to important variations in composition. To overcome this problem, the clotting activity of an enzyme must be defined with respect to an enzyme standard whose proteolytic activity will be determined on a reference peptide. That was

the purpose of the hexapeptide first proposed by Raymond et al. [10] and later by de Koning et al. [38] for chymosin and bovine pepsin.

Further, the clotting activities of calf rennet substitutes, such as the *Mucor* milk clotting proteases, whose characteristics (e.g. specificity [39—42]; proteolytic activity [43,44], dependence on pH and temperature (see Results)) are different from those of chymosin, must be assayed against an enzyme standard of the same nature tested on a standard peptide. In this connection, the results of our kinetic studies indicate that the synthetic hexapeptide of Raymond et al. [10] is a good substrate for *M. pusillus* and *M. miehei* aspartate proteases, since their k_{cat}/K_m ratio is twice that of chymosin. Besides, it is noticeable that both fungal proteases share a similar k_{cat}/K_m value as Morihara and coworkers [39,45] found, using the two following synthetic tetrapeptides:

Z—Phe$\overset{\downarrow}{-}$Leu—Ala—Ala ($k_{cat}/K_m \simeq 0.06$ mM$^{-1} \cdot$ s^{-1})

Z—Gly—Phe$\overset{\downarrow}{-}$Leu—Ala ($k_{cat}/K_m \simeq 0.12$ mM$^{-1} \cdot$ s^{-1})

which were poor substrates compared to the hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe. Therefore, the hexapeptide, which is also an excellent substrate for bovine and porcine pepsins [11], should be a useful tool for proteolytic activity measurements of most of the aspartate proteases and especially of milk clotting aspartate proteases.

In light of the effect of pH on the initial velocity of chymosins A and B, the pH optimum for crystalline chymosin is probably close to 4.0. This is in agreement with the results obtained by Raymond and Bricas (unpublished results) under the same conditions. These authors reported an initial velocity maximum for crystalline chymosin at around pH 4.3. However, de Koning et al. [38] found a pH optimum at 4.7 for chymosin as well as for bovine pepsin A, whereas Raymond [11] found for bovine pepsin A a pH optimum at about 3.5.

Based on the kinetic data reported in this paper and previous results [11,31], the aspartate proteases tested so far may be divided into two classes according to their k_{cat}/K_m ratios when assayed with the hexapeptide substrate. The first class, with k_{cat}/K_m ratios greater than 1000 mM$^{-1} \cdot$ s^{-1}, includes bovine and porcine pepsins and the second class, with k_{cat}/K_m ratios lower than 100 mM$^{-1} \cdot$ s^{-1}, includes *M. pusillus* and *M. miehei* proteases and chymosins. Foltmann [46] previously proposed a classification for mammalian gastric proteases that is based upon the pH optimum for activity. The proteolytic activities of bovine and porcine pepsins with respect to haemoglobin are optimal around pH 2.0 [47] whereas those for the other aspartate proteases that we have studied are optimal at about pH 3.5 [16,26,47,48]. In this connection, it is interesting that Foltmann [48] found that chymosins A, B and C had the same pH optima of around 3.5 with acid-denatured heamoglobin as a substrate. On the other hand, we found that chymosins A and B can be distinguished with respect to their pH optima for activity using the hexapeptide substrate; presumably the chymosins can be distinguished because they split only one peptide bond.

Clotting and proteolytic activities were obtained under rather similar experimental conditions. It is very attractive to try to correlate these activities.

Indeed, clotting activity was a rough expression of the enzymatic activity towards the single Phe_{105}-Met_{106} peptide bond in κ-casein. It appears that chymosins, which exhibit catalytic efficiencies similar to those of *M. pusillus* and *M. miehei* proteases towards the synthetic hexapeptide related to κ-casein (corresponding to amino acid sequences 103—108), have much greater clotting abilities than the fungal enzymes. The clotting activity/proteolytic activity ratios are about 6-fold higher for chymosins than for the fungal proteases. Kovacs-Proszt and Sanner [49] showed that it was necessary to use twice as much *M. pusillus* protease as chymosin to obtain the same clotting time, in agreement with our results. On the other hand, bovine pepsin A whose clotting activity/proteolytic activity ratio is about 25-fold lower than those obtained with chymosins, exhibits the same clotting activity and a higher proteolytic activity compared to the chymosins. Although this may seem inconsistant with a correlation between clotting activity and proteolytic activity it must be kept in mind first, that both activities were not determined at the same pH (which makes the correlation for bovine pepsin especially difficult) and second, that the hexapeptide is very different from κ-casein in structure. It would appear that although the four aspartate proteases examined in the present study have a high degree of sequence homology [46] and very similar tertiary structures [50] and although these proteases have a similar catalytic site (in which two aspartyl residues occurring in almost identical peptide sequences are involved [51]), they nevertheless possess extended active sites [52,53] whose detailed structures may differ in regions involved in secondary enzyme-substrate interactions. Thus, bovine pepsin, chymosin and *Mucor* proteases differ slightly in their proteolytic specificity on the β-chain of oxidized insulin [39,40,42,54,55]. Whereas chymosin and bovine pepsin cleave the Glu_{13}-Ala_{14} bond, *Mucor* proteases are not able to split this peptide bond.

The important differences observed between the action of milk clotting proteases on the hexapeptide compared to κ-casein might partly be ascribed to whether or not the whole active site of the enzyme, and especially the binding site, participate in the reaction. With a protein substrate such as κ-casein, which is preferentially hydrolysed at the Phe-Met bond, amino acid residues far away of this sensitive bond may play an important role in the interaction between the enzyme and the substrate. As a consequence of this, peptidase and proteinase activities must be distinguished. Even though the aspartate proteases examined in this paper share very similar molecular characteristic, they present important differences in enzymic properties (specificity, pH optimum, proteolytic and clotting activities) which may be attributed to the extent of the binding site. This is especially noticeable for chymosins A and B which seem to differ, as far as we know, by only a single substitution (Asp/Gly) at position 290 of the peptide chain [25]. The greater clotting activity of chymosin A compared to chymosin B towards κ-casein, as well as towards reconstituted skimmed milk [48], could be explained by the different pH optima exhibited by chymosins A and B.

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