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Differences in the P1′ substrate specificities of pepsin A and chymosin

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Porcine pepsin A and bovine chymosin are typical models of aspartic proteinases. The hydrolytic specificities of these proteinases, along with those of human pepsin A and monkey chymosin, were investigated with 29 peptide substrates that included various P1′ variants of seven parent peptides. From these peptides, AFPLEF↓FREL was preferred by pepsin A and chymosin, while its P1′ variant, AFPLEF↓EREL was preferred by bovine chymosin. Porcine and human pepsin A showed similar hydrolytic specificities, strongly preferring a hydrophobic/aromatic residue at P1′ of any type of peptide. This specificity is well explained by the very hydrophobic nature of the S1′ subsite that consists of Tyr^{189}, Ile^{213}, Ile^{300}, Met^{289}, Val/Leu^{291} and Leu^{298}. The first three residues are well conserved in pepsin family enzymes. Although bovine and monkey chymosin showed similar P1′ specificity, bovine chymosin preferred peptides having Glu at P1′, while monkey chymosin preferred peptides having Lys at P1′. The dual characteristics of chymosin are due to the occurrence of polar/charged residues in the S1′ subsite, such as Glu/Asp^{289}, Gln^{298} and Lys/Gln^{299}, which are different from the S1′ subsite of pepsin A. Molecular models suggest that Glu in position 289 of bovine chymosin and Asp in position 289 of monkey chymosin are responsible for the difference in P1′ specificities between the chymosins.

Keywords: aspartic proteinase/chymosin/pepsin A/proteolytic activity/substrate specificity.

Abbreviations: FGF, fibroblast growth factor; NT/NMN, neurotensin/neuromedin precursor; POMC, proopiomelanocortin.

Pepsins, the major proteolytic enzymes in vertebrate gastric juices, digest a variety of proteins and peptides at acidic pH (*1*). To date, five major types of pepsin have been identified (*2*, *3*). Pepsin A (E.C. 3.4.23.1) and chymosin (E.C. 3.4.23.4) are two typical pepsins that have been studied extensively as models of aspartic proteinases. Chymosin is also important in the cheese making industry. Pepsin A is known to be widely distributed in adult vertebrates, whereas chymosin is distinct in that it is expressed predominantly at fetal and neonatal stages, and it is known to be essential for neonatal milk digestion (*4*, *5*). These two enzymes have diverged from a common ancestor during the evolution of vertebrates (*3*). Similarities in the primary structures of pepsin A and chymosin range from 50% to 60% between mammalian species (*3*); however, the essential tertiary structures are well conserved (*6*, *7*). The proteolytic specificities of pepsin A and chymosin have been investigated using various substrates including proteins and peptides. Extensive studies have established that porcine pepsin A hydrolyses peptide bonds that connect bulky hydrophobic/aromatic residues, such as Phe-Trp, Phe-Tyr and Phe-Phe (*8*, *9*), and accommodates seven-residue peptides in its active site (*10*). Bovine chymosin has been frequently studied using milk caseins as substrates, and it has been found that the enzyme prefers similar hydrophobic/aromatic dipeptide sequences at P1–P1′, especially cleaving the Phe^{105}-Met^{106} bond of κ-casein as the initiation step of milk coagulation (*5*). In some cases, chymosin has been shown to accommodate charged or polar residues at P1′, unlike pepsin A (*11–13*). It is, however, the proteolytic specificity of chymosin remains to be clarified in order to understand its structure and functional relationship in detail.

The use of peptide substrates for elucidating proteolytic specificity is advantageous because systematic changing of the peptide residues enables the clarification of unique specificities. With a set of synthetic chromogenic peptides, Dunn *et al.* (*14–16*) have elucidated the details of favourable or unfavourable amino acids from the P4 position through to the P3′ position of substrates for porcine pepsin A, showing for instance that Pro at P4, hydrophobic residues at P3 and P3′, and Ala/Val at P2′ are preferred. Thus, KPAEFFRL has been proposed as good pepsin-A substrate (tentatively termed 'pepsin substrate' in the present report). Apart from these synthetic oligopeptides, some bioactive peptides, such as POMC 165–174 and NT/NMN 142–151, have also been shown to be good pepsin substrates (*17*, *18*).

In a previous report, we investigated the substrate specificities of human pepsin A and monkey chymosin, and we found that in marked contrast to human pepsin

A, monkey chymosin has a high preference for Lys at P1′ (*18*). The residues of the S1′ subsite that are involved in the unique specificities of monkey chymosin were addressed with site-directed mutagenesis, showing that charged/polar residues are essential for this unique specificity (*18*). It is appropriate to clarify whether the differences in specificities of pepsin A and chymosin are common among mammalian pepsins.

In the present study, porcine pepsin A and bovine chymosin were chosen along with human pepsin A and monkey chymosin. Twenty-nine peptides, which were different at the P1′ site, were used. The results showed that porcine and human pepsin A have quite similar specificities, preferring a hydrophobic/aromatic residue at P1′. Although bovine and monkey chymosin showed preferences similar to porcine and human pepsin A, they also liked charged residues at P1′. The similarities and differences between the four enzymes are discussed together with the tertiary structures of their S1′ subsites.

Materials and methods

Materials

Porcine pepsin A and bovine chymosin were obtained from Sigma Chemical Co. Human pepsin A and monkey chymosin were prepared by activation of the respective recombinant proenzymes. Peptides were synthesized by Sigma Genosys, Ishikari, Japan. All other chemicals were of reagent or analytical grade.

Assay of proteolytic activity

Proteolytic activity was determined by using solutions of ~2% haemoglobin and casein as substrates at pH 2.0 and 3.5, and 37°C (*19*). The amount of peptides released was determined by a fluorometric assay with fluorescamine with leucine as a standard (*20*).

Assay of peptide hydrolysing activity

Hydrolysis of peptides was assessed by methods described previously (*18*). In brief, the reaction mixture contained 0.2 M sodium formate buffer, pH 4; 50 μM of peptide and an appropriate amount of enzyme. The total volume was 20 μl. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 80 μl of 3% perchloric acid. Following removal of any precipitated material by centrifugation, the reaction mixtures were subjected to HPLC on an ODS-120T column (0.46 cm i.d. × 25 cm) (Tosoh Corp., Tokyo, Japan) that had been equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of acetonitrile from 0% to –60% (v/v) over the course of 24 min in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. Quantification of the peptides was carried out using their peak areas. The relative absorption coefficient of each peptide at 214 nm was calculated using Stephenson's and Kenny's equation (*21*).

Determination of kinetic parameters

The reaction mixture for determining the kinetic parameters for the cleavage of peptides contained 200 mM sodium formate buffer, pH 4; 0.5–5 ng enzyme and 10–100 μM substrate peptide in a total reaction volume of 50 μl. The reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by the addition of 50 μl of 3% perchloric acid. The reaction was carried out in triplicate for each substrate concentration. Plots of $1/v$ against 1/[S] (Lineweaver-Burk) permitted the fitting of a straight line by linear regression, resulting in the determination of K_m and V_{max}. By using the equation for $V_{max}/[E]_0$, k_{cat} was obtained. When a peptide was cleaved at two different sites, K_m values for these sites were calculated on the assumption that the respective counter cleavage sites function competitively (*22*).

Molecular modelling

Tertiary structural models of pepsin A and chymosin complexed with peptide substrates were constructed using Modeller version 9v6 (*23*) and the crystal structures of the complex between human pepsin A and pepstatin (*24*), porcine pepsin A (*25–27*) and bovine chymosin (*28*) as the initial models. Images were created using RASMOL version 2.5.

Results

Substrate specificity of pepsin A

The proteolytic activities of pepsin A and chymosin were first assayed with the conventional protein substrates, haemoglobin and casein. Haemoglobin was a better substrate than casein, being hydrolysed rapidly. Although pepsin A showed higher activity at pH 2.0 than at pH 3.5, and chymosin exhibited the opposite, the results showed that both pepsin A and chymosin have sufficient general proteolytic activities. Hydrolytic specificity was investigated with 7–10-residue oligopeptides as substrates. Twenty-nine peptides were used, which included seven parent peptides and their P1′ variants. Hydrolysis was done at pH 4.0, since the optimal pH for peptide hydrolysis has been shown to be 4.0, being different from the case of protein hydrolysis (*18*). Although 12 of them were examined with human pepsin A and monkey chymosin in our previous study (*18*), most of the peptides were hydrolysed here for the first time, especially in the cases of porcine pepsin A and bovine chymosin. The parent peptides were pepsin substrate (PKAEFFRL) (*16*), POMC 165–174 (AFPLEF KREF), NT/NMN 142–151 (KIPYILKRQL), basic FGF 110–118 (KYSSWYVAL), substance P (RPKPQQFFGLM$_{NH2}$), dynorphin A (YGGFLRR) and κ-casein 100–108 (HPHLSFMAI). Hydrophobic/aromatic residues such as Phe and Ile, and charged residues such as Lys and Glu were included as P1′ residues in most of the peptide groups since the accommodation of these types of residues has been shown to be significantly affected by the structure of the S1′ subsite (*18*). The hydrolytic activities of porcine and human pepsin A, and bovine and monkey chymosin against these peptides are summarized in Table 2. The results show that porcine pepsin A efficiently hydrolysed peptides having hydrophobic/aromatic

Table 1. Hydrolytic activities of pepsin A and chymosin against protein substrates.

Substrate and pH	Hydrolytic activity [nmol Leu min^{-1} (μg protein)$^{-1}$][a]			
	Pepsin A		Chymosin	
	Porcine	Human	Bovine	Monkey
Haemoglobin				
pH 2.0	**400 ± 20**	**318 ± 18**	155 ± 11	181 ± 30
pH 3.5	171 ± 30	215 ± 43	**363 ± 56**	**285 ± 17**
Casein				
pH 2.0	**166 ± 8**	**99 ± 3**	28 ± 6	29 ± 4
PH 3.5	5.1 ± 0.3	3.2 ± 0.3	**7.3 ± 1.9**	**11 ± 1**

[a]When compared between pepsins A and chymosins, the values that were significantly larger ($P < 0.05$) than those of counter enzymes are shown in bold.

Table 2. Hydrolytic activities of pepsin A and chymosin against typical peptide substrates.

Peptide	Sequence and cleavage site[b]	Hydrolytic activity [nmol min^{-1} (μg protein)$^{-1}$][a]			
		Pepsin A		Chymosin	
		Porcine	Human	Bovine	Monkey
Pepsin substrate					
Parent peptide	KPAEF↓FRL	**22 ± 2**	**15 ± 1**	4.5 ± 0.3	1.4 ± 0.1
Lys^{6} variant	KPAEF↓**K**RL	0.22 ± 0.01	uc[c]	uc	**0.31 ± 0.02**
Glu^{6} variant	KPAEF↓**E**RL	**1.5 ± 0.1**	**0.72 ± 0.10**	0.12 ± 0.02	uc
Thr^{6} variant	KPAEF↓**T**RL	**1.7 ± 0.2**	**1.2 ± 0.2**	0.09 ± 0.01	uc
Gly^{6} variant	KPAEF↓**G**RL	uc	uc	uc	uc
Ala^{6} variant	KPAEF↓**A**RL	**6.3 ± 0.2**	**2.9 ± 0.1**	0.28 ± 0.04	uc
Val^{6} variant	KPAEF↓**V**RL	**13 ± 1**	**4.9 ± 1.0**	0.83 ± 0.33	0.24 ± 0.02
Leu^{6} variant	KPAEF↓**L**RL	**17 ± 1**	**6.6 ± 0.1**	0.85 ± 0.09	0.23 ± 0.01
Ile^{6} variant	KPAEF↓**I**RL	**27 ± 3**	**9.6 ± 1.4**	1.7 ± 0.5	0.59 ± 0.03
κ-Casein 100–108					
Parent peptide	HPHLSF↓MAI	**89 ± 7**	**24 ± 5**	14 ± 2	0.91 ± 0.27
Glu^{106} variant	HPHLSF↓**E**AI	**7.2 ± 0.4**	0.86 ± 0.21	3.1 ± 0.2	0.31 ± 0.10
Dynorphin A 1–7[d]					
Ile^{3}Phe^{7} variant	YGI**F**↓LR**F**	**13 ± 1**	**0.72 ± 0.04**	0.34 ± 0.01	0.02 ± 0.01
Ile^{3}Lys^{5}Phe^{7} variant	YGI**F**↓**K**R**F**	1.3 ± 0.2	0.06 ± 0.01	uc	0.04 ± 0.01
Basic FGF 110–118					
Parent peptide	KYSSW↓YVAL	**17 ± 2**	**6.7 ± 0.4**	4.1 ± 0.3	0.17 ± 0.03
Lys^{115} variant	KYSSW↓**K**VAL	0.15 ± 0.01	uc	**0.60 ± 0.12**	0.06 ± 0.01
Glu^{115} variant	KYSSW↓**E**VAL	0.23 ± 0.01	0.70 ± 0.02	**3.0 ± 0.4**	uc
Substance P					
Parent peptide	RPKPQQF↓FGLM$_{NH2}$	**2.5 ± 0.3**	**4.9 ± 0.5**	0.51 ± 0.03	0.15 ± 0.01
Lys^{8} variant	RPKPQQF↓**K**GLM	uc	uc	**0.04 ± 0.01**	**0.20 ± 0.01**
POMC 165–174					
Parent peptide	AFPLEF↓KREL	1.8 ± 0.1	0.86 ± 0.18	**16 ± 2**	**26 ± 2**
Parent peptide	AFPLE↓FKREL	2.4 ± 0.1	2.0 ± 0.3	uc	uc
Glu^{171} variant	AFPLEF↓**E**REL	6.3 ± 1.4	1.5 ± 0.1	**58 ± 7**	**11 ± 2**
Glu^{171} variant	AFPLE↓F**E**REL	2.6 ± 0.4	uc	uc	uc
Ile^{171} variant	AFPLEF↓**I**REL	147 ± 5	56 ± 5	**323 ± 33**	79 ± 5
Phe^{171} variant	AFPLEF↓**F**REL	145 ± 16	82 ± 6	**351 ± 30**	130 ± 6
NT/NMN 142–151					
Parent peptide	KIPYIL↓KRQL	1.9 ± 0.5	0.93 ± 0.12	1.6 ± 0.3	**15 ± 2**
Arg^{148} variant	KIPYIL↓**R**RQL	1.9 ± 0.4	0.78 ± 0.03	1.1 ± 0.1	**11 ± 1**
Glu^{148} variant	KIPYIL↓**E**RQL	3.6 ± 0.5	2.4 ± 0.1	**5.4 ± 0.6**	**13 ± 2**
Ser^{148} variant	KIPYIL↓**S**RQL	2.3 ± 0.4	2.0 ± 0.4	2.7 ± 0.4	**6.7 ± 0.4**
Ala^{148} variant	KIPYIL↓**A**RQL	11 ± 1	14 ± 3	7.8 ± 1.4	11 ± 1
Ile^{148} variant	KIPYIL↓**I**RQL	**33 ± 4**	7.2 ± 0.9	16 ± 1	7.6 ± 0.5
Phe^{148} variant	KIPYIL↓**F**RQL	9.0 ± 0.7	10 ± 1	3.1 ± 0.3	**21 ± 2**
Phe^{148} variant	KIPYIL**F**↓RQL	9.6 ± 1.5	3.3 ± 0.1	9.2 ± 0.6	**61 ± 3**
Trp^{148} variant	KIPYIL↓**W**RQL	2.1 ± 0.5	3.3 ± 1.1	2.1 ± 0.1	3.4 ± 0.8

[a]When compared between pepsins A and chymosins, the values that are significantly larger ($P < 0.05$) than those of counter enzymes are shown in bold. [b]In the peptide sequences, replaced residues are shown in bold. [c]uc indicates that the peptide was uncleaved or the rate of its hydrolysis gave a value <0.01 nmol min^{-1} (μg protein)$^{-1}$. [d]The sequence of the parent peptide is YGGFLRR, which is not hydrolyzed by pepsins A and chymosin.

residues at P1′. Phe/Ile^{171}-POMC 165–174 was the best substrate. Similar results were obtained with human pepsin A, except that dynorphin-derived peptides were poor substrates. It is clear that porcine and human pepsin A both strongly prefer a hydrophobic/aromatic residue at P1′ in any type of peptide, whereas charged residues at P1′ were disliked.

Substrate specificity of chymosin

Contrary to the pepsin A results, pepsin substrate (KPAEFFRL) was not a good substrate for either chymosin. The most remarkable result was that bovine chymosin and monkey chymosin very rapidly hydrolysed Phe/Ile^{171}-POMC 165–174. The bovine chymosin hydrolysis rate of Phe^{171}-POMC 165–174 was the highest between the peptides examined in the present study. Indeed, the rate was 2-fold faster than that by porcine pepsin A. κ-Casein 100–108 and NT/NMN 142–151 were also good substrates for bovine and monkey chymosin, respectively. Since these peptides were less accommodated than their respective counter chymosin, species-specific characteristics of chymosin were anticipated. Other substrates including substance P and dynorphin-derived peptides were poor substrates, although basic FGF 110–118 was moderately hydrolysed by bovine chymosin. These results show that chymosin and pepsin A preferentially hydrolyse a hydrophobic/aromatic residue at P1′.

It is noteworthy that bovine and monkey chymosin also efficiently hydrolysed peptides having Lys or Glu at P1′. This was typical in the cases of POMC

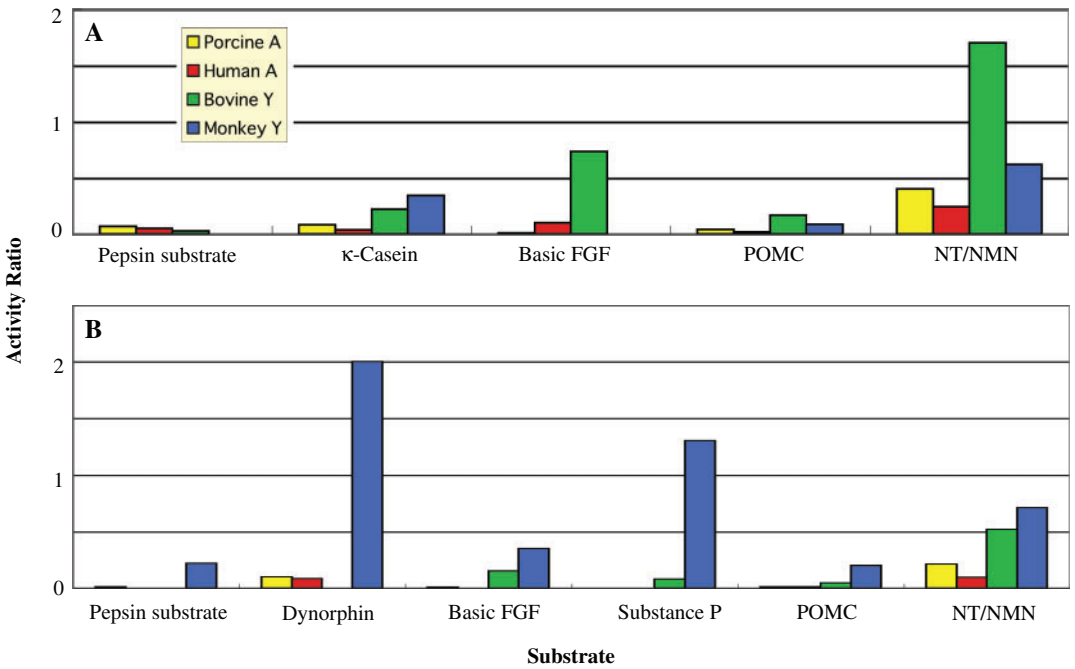

Fig. 1 Activity ratios of the hydrolyzing activities of pepsin A and chymosin against peptides having Glu (A) and Lys (B) at P1′ to the activities against peptides having Phe (pepsin substrate, substance P, POMC 165–174 and NT/NMN 142–151), Met (κ-casein), Leu (dynorphin A 1–7) and Tyr (basic FGF 110–118) at P1′. A and Y stand for pepsin A and chymosin, respectively.

165–174 (AFPLEF↓KREL), Glu[171]-POMC 165–174 (AFPLEF↓EREL), NT/NMN 142–151 (KIPYIL↓KRQL), Glu[148]-NT/NMN 142–151 (KIPYIL↓ERQL) and Glu[115]-basic FGF 110–118 (KYSSW↓EVAL). When the ratios of activity against peptides having Glu/Lys at P1′ are compared to the ratios of activity against peptides having Phe or another hydrophobic residue, the results clearly show that, except for pepsin substrate, peptides having charged residues at P1′ were hydrolysed more efficiently by chymosin than by pepsin A (Fig. 1). It is also clear that bovine chymosin preferred Glu at P1′, whereas monkey chymosin preferred Lys at P1′.

Kinetic constants

Comparison of the Michaelis–Menten kinetics of the typical substrates POMC 165–174 and its variants provided k_{cat}/K_m values in line with the rates for hydrolysis, as shown in Table 3. In the case of hydrolysis of Phe[171]-POMC 165–174 by porcine pepsin A, the K_m and k_{cat} values were 170 μM and 472 s^{-1}, respectively. The k_{cat} value decreased significantly with the variant peptides having Lys and Glu at P1′, resulting in a lowering of the hydrolytic rate. The hydrolysis of Phe[171]-POMC 165–174 by bovine chymosin, which is the best substrate-enzyme combination, produced K_m and k_{cat} values of 41 μM and 290 s^{-1}, respectively. The low K_m value compared to that of porcine pepsin A contributed to the highest hydrolytic rate. When P1′ residue was replaced with Glu or Lys, an increase of K_m and a decrease of k_{cat} values were observed resulting in a lowering of the hydrolysis rate. It is noteworthy that the high k_{cat} value of the Glu[171]-variant was obvious, showing the high turnover of the enzyme–substrate complex. The high k_{cat} value contributed clearly to the preferential hydrolysis of Glu[171]-POMC 165–174 by bovine chymosin.

Discussion

Porcine and human pepsin A showed similar specificities against the peptides used in the present study. Both preferred a hydrophobic/aromatic residue at P1′. Although bovine and monkey chymosin shows P1′ specificities similar to those of pepsin A, they also showed a preference for charged residues like Glu/Lys at P1′. Such unique P1′ specificity has not been established till date, except that a Lys preference at P1′ has been reported in monkey chymosin (*18*). The specificities of pepsin A and chymosin are discussed here together with the tertiary structure of the S1′ subsites of the respective enzymes. First, let us examine a modelled structure of the S1′ subsite of porcine pepsin A accommodating Phe[171]-POMC 165–174. The S1′ subsite of porcine pepsin A consists of six residues. Three of the residues are Tyr[189], Ile[213] and Ile[300], which are very conservative, while the three residues are Met[289], Val[291] and Leu[298], which are variable between pepsins (*3*, *7*). The variable residues are located in the S1′ loop structure (residues 289–299) (Fig. 2). Thus, the S1′ subsite of porcine pepsin A is very hydrophobic, preferring hydrophobic/aromatic residues (*25–27*). These subsite residues are arranged like a sandwich: one slice of bread is small consisting of only Tyr[189] and the other slice of bread is large consisting of residues 213, 289, 291, 298 and 300 (Fig. 3). A hydrophobic/aromatic residue at P1′ of a substrate locates

Table 3. Kinetic constants for the hydrolysis of POMC 165–174 and its variants by pepsin A and chymosin.

Peptide	Pepsin A		Chymosin	
	Porcine	Human	Bovine	Monkey
POMC 165–174 Parent peptide (AFPLEF↓KREL)				
K_m	0.019 ± 0.005	0.034 ± 0.003	0.18 ± 0.002	0.38 ± 0.04
k_{cat}	2.7 ± 0.5	1.4 ± 0.2	29 ± 3	219 ± 24
k_{cat}/K_m	140 ± 30	41 ± 2	**160 ± 2**	**590 ± 20**
(AFPLE↓FKREL)				
K_m	0.022 ± 0.005	0.029 ± 0.003		
k_{cat}	3.7 ± 0.7	2.7 ± 0.2	uc	uc
k_{cat}/K_m	**170 ± 30**	**93 ± 5**		
Glu[171] variant (AFPLEF↓**E**REL)				
K_m	0.40 ± 0.04	0.30 ± 0.03	3.3 ± 1.0	0.30 ± 0.07
k_{cat}	45 ± 4	8.9 ± 2.0	2600 ± 500	63 ± 13
k_{cat}/K_m	110 ± 10	29 ± 2	**790 ± 200**	**210 ± 20**
(AFPLE↓F**E**REL)				
K_m	0.60 ± 0.12			
k_{cat}	30 ± 6	uc	uc	uc
k_{cat}/K_m	**50 ± 2**			
Ile[171] variant (AFPLEF↓**I**REL)				
K_m	0.098 ± 0.013	0.079 ± 0.019	0.079 ± 0.007	0.044 ± 0.005
k_{cat}	203 ± 14	56 ± 5	427 ± 19	82 ± 2
k_{cat}/K_m	2100 ± 100	730 ± 110	**5400 ± 300**	1900 ± 200
Phe[171] variant (AFPLEF↓**F**REL)				
K_m	0.17 ± 0.04	0.084 ± 0.008	0.041 ± 0.002	0.027 ± 0.001
k_{cat}	472 ± 55	85 ± 4	290 ± 4	68 ± 4
k_{cat}/K_m	2800 ± 400	1000 ± 100	**7100 ± 200**	2500 ± 100

The units of K_m, k_{cat} and k_{cat}/K_m are mM, s^{-1} and $mM^{-1}s^{-1}$, respectively. In the peptide sequence, the replaced residues are shown in bold. When k_{cat}/K_m values were compared between pepsins A and chymosins, the values that are significantly larger than those of counter enymes are shown in bold. The term uc indicates that the peptide was uncleaved or that the rate of its hydrolysis gave a value <0.01 nmol min^{-1} (μg protein)[1].

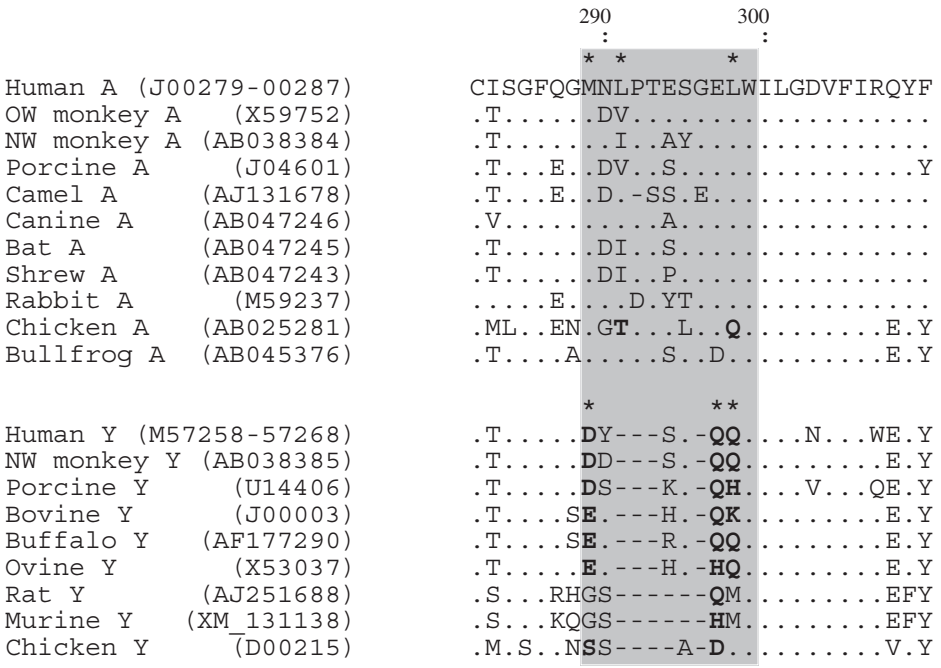

Fig. 2 Primary structures of the variable region of the S1′ subsites of pepsin A and chymosin (denoted by A and Y, respectively) from various vertebrate sources. Porcine pepsin A numbering is used. Dots indicate amino acids that are identical to the human sequence. Deleted residues are shown by short bars. Residues in the 289–299 region forming the loop structure that is part of the S1′ subsite are shaded. Asterisks show positions that possibly contact the P1′ residue of a substrate. Charged or polar residues at these positions are indicated with bold. GenBank/EMBL/DDBJ accession numbers are given in parentheses. OW and NW stand for Old World and New World, respectively.

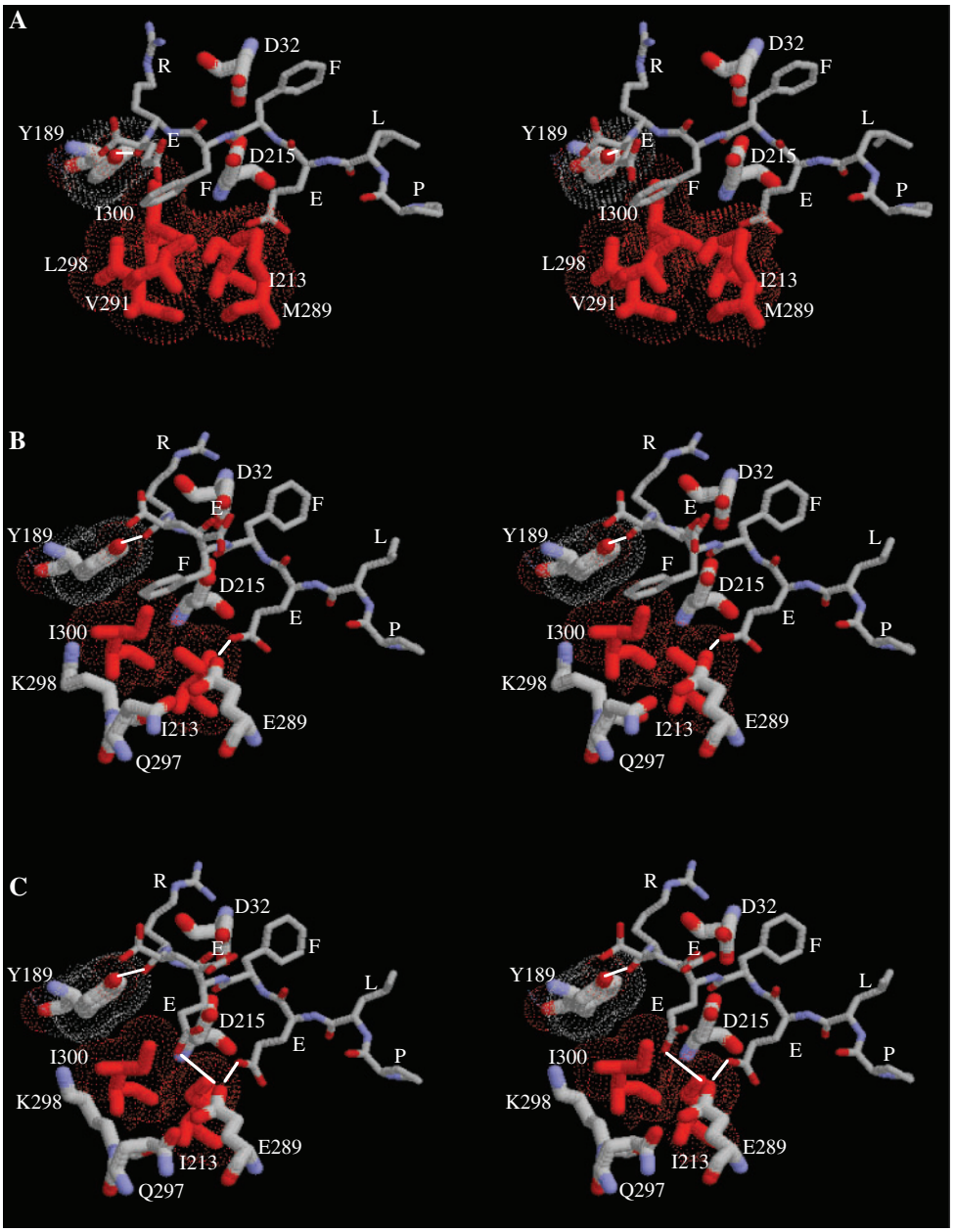

Fig. 3 Steroviews of the spatial arrangement of the residues in the putative S1′ subsite and a substrate, and catalytic aspartates. (A) Porcine pepsin A in complex with PAEFFRE (Phe^{171}-POMC 165–174). (B) and (C) Bovine chymosin in complex with PAEFFRE (Phe^{171}-POMC 165–174) (B) and PAEFERE (Glu^{171}-POMC 165–174) (C). Hydrophobic residues are shown in red wireframes. Other residues are shown by wireframes coloured grey, red and light blue for carbon, oxygen and nitrogen, respectively. Because van der Waals surface forces are known to have significant roles in the attraction of hydrophobic residues, they are given here as dots for hydrophobic residues and Tyr^{189} in the putative S1′ subsite. Possible hydrogen bonds between the S1′ subsite residues and the substrate are shown by white bars. Porcine pepsin-A numbering is used.

between these two 'slices of bread'. For example, Phe at P1′ is accepted just like ham between two slices of bread. Since the amino acid replacement of the S1′ subsite between porcine and human pepsin A occurs only at position 291 with very similar residues (Val and Leu in porcine and human enzymes, respectively), a difference in the sandwich effect between these two types of pepsin A is unexpected, supporting the similar S1′ specificities between them.

The S1′ subsite of chymosin is less hydrophobic than that of pepsin A (*28*, *29*). Although Tyr^{189}, Ile^{213} and Ile^{300} are well conserved in chymosin, other residues are largely different from those of pepsin A (Fig. 2). The 289–299 loop is 4–6 residues shorter that that of pepsin A and mainly consists of charged and polar residues, such as Glu^{289}, Gln^{297} and Lys^{298} in bovine chymosin and Asp^{289}, Gln^{297} and Gln^{298} in monkey chymosin. Thus, the S1′ subsite of chymosin has dual characters; it is partly hydrophobic and partly hydrophilic. When the most preferred substrate, Phe^{171}-POMC 165–174, approaches the active site of bovine chymosin, Tyr^{189}, Ile^{213} and Ile^{300} are thought to form a hydrophobic pocket to accommodate Phe at P1′ (Fig. 3B). The pocket no longer has a sandwich structure of hydrophobic/aromatic residues but consists of two different cores of residues,

i.e. hydrophobic and hydrophilic cores. Since the size of the hydrophobic core is much smaller than that of porcine pepsin A, the S1′ specificity for hydrophobic/aromatic residues is expected to be weak, as evidenced by occasionally lower activity against peptides having a hydrophobic/aromatic residue at P1′ (Table 2). High activity against Phe^{171}-POMC 165–174 is obviously exceptional, and the occurrence of interactions other than a hydrophobic one is anticipated to occur between the substrate and the enzyme. One possible interaction might be the hydrogen bond between O^{ε} of Glu^{289} and O^{ε} of Glu at P3, which contributes to the increase in affinity of the substrate with the enzyme (Fig. 3B).

Higher susceptibility of bovine and monkey chymosin for Glu/Lys at P1′ is clearly due to the occurrence of charged/polar residues at the S1′ subsite. The replacement of these residues with hydrophobic ones has been shown to increase the preference for a hydrophobic/aromatic residue at P1′ (*18*). Bovine chymosin showed a higher preference for Glu at P1′ than monkey chymosin and both porcine and human pepsin A (Fig. 1). The molecular model shows the possibility that O^{ε} of Glu^{289} plays an important role in generating hydrogen bonds with O^{ε} of Glu at P1′ along with O^{ε} of Glu at P3 (Fig. 3C). Lys might be accommodated less preferentially at P1′than Glu since it has a long side chain that may make it unable to correctly form hydrogen bonds with O^{ε} of Glu^{289}. Since Glu^{289} is replaced with Asp in monkey chymosin, the strong hydrogen-bonding interaction of O^{δ} of Asp289 and N^{ζ} of Lys at P1′ is expected to accommodate a peptide having Lys at P1′ (Fig. 1) (*18*). Other possible hydrogen bonding interactions between bovine chymosin and Glu^{171}-POMC 165–174 are expected to be those between N^{ζ} of Lys^{298}/N^{ε} of Gln^{297} and O^{ε} of Glu at P1′.

When compared, the k_{cat} values of chymosin are higher than those of pepsin in most substrates, being especially high in the case of Glu^{171}-POMC 165–174 hydrolysis by bovine chymosin. Such a high value contributes to the preferential hydrolysis of peptides having Glu at P1′, due to the rapid dissociation of the enzyme–substrate complex. Structural flexibility of the active site has been shown to affect k_{cat} (*30*). Although the hydrophobic sandwich structure of the S1′ subsite of pepsin A is thought to be very rigid, the S1′ subsite of chymosin might be rather flexible judging from its incomplete hydrophobic-core structure. Thus, the high k_{cat} value of chymosin might be due to the enzyme-specific flexible structure of the S1′ subsite.

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Conflict of interest
None declared.

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