Role of S′1 Loop Residues in the Substrate Specificities of Pepsin A and Chymosin†

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ABSTRACT: Proteolytic specificities of human pepsin A and monkey chymosin were investigated with a variety of oligopeptides as substrates. Human pepsin A had a strict preference for hydrophobic/aromatic residues at P′1, while monkey chymosin showed a diversified preferences accommodating charged residues as well as hydrophobic/aromatic ones. A comparison of residues forming the S′1 subsite between mammalian pepsins A and chymosins demonstrated the presence of conservative residues including Tyr^{189}, Ile^{213}, and Ile^{300} and group-specific residues in the 289−299 loop region near the C terminus. The group-specific residues consisted of hydrophobic residues in pepsin A (Met^{289}, $Leu/Ile/Val^{291}$, and Leu^{298}) and charged or polar residues in chymosins (Asp/Glu^{289} and $Gln/His/Lys^{298}$). Because the residues in the loop appeared to be involved in the unique specificities of respective types of enzymes, site-directed mutagenesis was undertaken to replace pepsin-A-specific residues by chymosin-specific ones and vice versa. A yeast expression vector for glutathione-*S*-transferase fusion protein was newly developed for expression of mutant proteins. The specificities of pepsin-A mutants could be successfully altered to the chymosin-like preference and those of chymosin mutants, to pepsin-like specificities, confirming residues in the S′1 loop to be essential for unique proteolytic properties of the enzymes. An increase in preference for charged residues at P′1 in pepsin-A mutants might have been due to an increase in the hydrogen-bonding interactions. In chymosin mutants, the reverse is possible. The changes in the catalytic efficiency for peptides having charged residues at P′1 were dominated by k_{cat} rather than K_m values.

Pepsins, the major proteolytic enzymes in vertebrate gastric juices, digest a variety of proteins at acidic pH. To date, five major types of pepsin have been identified: pepsin A, pepsin B, gastricsin (pepsin C), chymosin, and pepsin F (*1*−*3*). The term "pepsin" has been used as a generic name for these enzymes (*4*), and the five types are known to have diverged from a common ancestor during the evolution of vertebrates (*3*). Their expression in the stomach differs between vertebrate species (*2*, *3*). For instance, although pepsin A is known to be widely distributed in vertebrates, it is absent in rodents. Chymosin is distinct in that it is expressed predominantly at fetal and neonatal stages (*5*−*7*) and is known to be essential for milk digestion by neonates. The diversity of pepsins suggests that each type of pepsin possesses unique actions in efficient digestion of food proteins in vertebrate species or during development.

Pepsin specificity has been investigated using various substrates, including proteins and peptides. Extensive studies with porcine pepsin A have established this enzyme as a typical model for analyzing structure and function of proteolytic enzymes (*8*−*10*). Earlier studies on pepsin-A specificity were conducted with synthetic peptides by Fruton and his colleagues, who clarified that it hydrolyzes peptide bonds that connect bulky hydrophobic/aromatic residues, such as Phe-Trp, Phe-Tyr, and Phe-Phe (*8*), and accommodates seven-residue peptides in its active site (*11*). A detailed survey of pepsin cleavage sites in 177 proteins has confirmed the occurrence of hydrophobic combinations in the cleavage sites (*12*). Specificity of bovine chymosin has also been studied in detail because it is an important enzyme for cheese making. Although chymosin is reported to resemble pepsin A in hydrolyzing hydrophobic peptide bonds (*2*, *3*), distinct differences remain to be clarified. With a set of synthetic chromogenic peptides, Dunn et al. (*13*−*15*) have elucidated the details of favorable or unfavorable amino acids at P4 through P′3 positions of substrates for pepsin A and chymosin, showing for instance that Pro at P4, hydrophobic residues at P3 and P′3, and Ala/Val at P′2 are preferred. However, these chromogenic synthetic peptides contained a fixed residue, p-NO_2Phe, at P′1 sites and thus restricted the analysis of the P′1 site specificity.

In parallel with protein and peptide hydrolytic analyses, site-directed mutagenesis has also been applied recently to explore pepsin specificity. X-ray structural studies suggest that pepsin-family members have a large active-site cleft in the center of the enzyme that contains two catalytic-site aspartates and several hydrophobic residues in the neighborhood of the catalytic residues (*16*−*20*). The cleft is sufficiently long to accommodate a seven-residue substrate, constituting S4−S′3. Because subsite-forming residues are thought to have essential roles in catalysis, they have been

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studied with site-directed mutagenesis, especially the S1 subsite of porcine pepsin A and bovine chymosin (*21*−*26*). Although the S′1 subsite is also thought to be a major determinant of specificity, its targeted mutagenesis was a desirable step forward.

Previously, we have shown a variety of oligopeptides including commercially available and synthetic examples to be useful as substrates in the analysis of the proteolytic specificities of aspartic proteinases (*27*, *28*). In the present study, I compared proteolytic specificities of human pepsin A and monkey chymosin, using oligopeptide substrates. It was found for the first time that chymosin has a high preference for charged residues such as Lys at P′1, in marked contrast to pepsin A. Because this different specificity for P′1 was thought to be essential, I then undertook to address the residues of the S′1 subsite that are involved in the unique specificities of pepsin A and chymosin, with site-directed mutagenesis. A yeast expression vector was newly developed to express wild-type and mutant enzymes. The results of mutagenesis show that residues 289, 291, and 298 of pepsin A and residues 289 and 298 of chymosin are involved in the determination of P′1 specificity.

EXPERIMENTAL PROCEDURES

Materials. The cloning vectors, pYES2, pGEX-4T-1, and pGEM-T Easy were purchased from Invitrogen Corp. Carlsbad, CA, Amersham Biosciences Corp., Piscataway, NJ, and Promega Corp., Madison, WI, respectively. QuickChange site-directed mutagenesis kit were obtained from Stratagene, La Jolla, CA; Expand enzyme mix, from Roche Diagnostics GmbH, Mannheim, Germany; Y-PER yeast protein extraction reagent, from Pierce Biotech. Inc., Rockford, IL; and glutathione Sepharose 4B and SP-Sephadex, from Amersham Biosciences. Peptides were purchased from Peptide Institute, Inc., Minoh, Japan, and Bachem AG, Bubendorf, Switzerland, except for commercially unavailable peptides, which were synthesized by Sigma Genosys Japan, Ishikari, Japan. A cDNA library of the human stomach was obtained from Takara Bio Inc., Otsu, Japan. Human pepsin A (*29*) and monkey chymosin (*30*) were purified as described previously. All other chemicals were of reagent or analytical grade.

Assay of Peptide-Hydrolyzing Activity. Hydrolysis of peptides was based on the methods described in our previous reports (*27*, *28*). In brief, the reaction mixture contained 0.2 M buffer at an appropriate pH, 50 μM of peptide, and an appropriate amount of enzyme. The total volume was 20 μL. After incubation at 37 °C for 1−6 h, the reaction was stopped by the addition of 80 μL of 3% perchloric acid. After removal of any precipitated material by centrifugation, each reaction mixture was subjected to HPLC on a column (0.46 cm inside diameter × 25 cm) of ODS-120T (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 28 min in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min. Each peptide was subjected to amino acid analysis and determination of the N- and/or C-terminal sequences with aminopeptidase M and/or carboxypeptidase Y to determine the sites of cleavage. Quantification of peptides was carried out using their peak areas. Relative absorption coefficient at 214 nm of each peptide was calculated from the equation of Stephenson and Kenny (*31*).

Determination of Kinetic Parameters. The reaction mixture for determining kinetic parameters for the cleavage of peptides contained 0.2 M sodium formate buffer at pH 4, 0.5−5 ng of enzyme, and 10−100 μM substrate peptide in a total reaction volume of 50 μL. The mixture was incubated at 37 °C for 30 min and stopped by the addition of 50 μL of 3% perchloric acid. The reaction was carried out 3 times 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}. k_{cat} was obtained with the equation of $V_{max}/[E]_0$.

Cloning of cDNAs for Human Pepsinogen A and Monkey Prochymosin and Site-Directed Mutagenesis. A DNA fragment including the coding sequence for human pepsinogen A was amplified by PCR using a plasmid of the human stomach cDNA library as the template and Expand enzyme mix as a DNA polymerase mixture. Upper and lower primers were 5′-CTCGAGAAAAGAATCATGTACAAGGTCCCCCTCATC and 5′-TCTAGATTTAGCCACGGGGGCCAGGCCGAC, respectively, which had *Xho*I and *Xba*I sites at the respective 5′ termini. The product was ligated into the TA cloning site of pGEM-T Easy vector. A cDNA for monkey prochymosin was molecular-cloned previously from the common marmoset (*Callithrix jacchus*) (*30*). To introduce *Xho*I and *Xba*I sites into the 5′ and 3′ termini of the coding sequence, PCR amplification was carried out with 5′-CTCGAGGCCAGTGGAATTGTCAGGAT and 5′-TCTAGATCAGATTGCCTTGGCCAGCC primers using a prochymosin cDNA plasmid as a template. The product was also ligated into the pGEM-T Easy vector.

Site-directed mutagenesis of human pepsinogen A and monkey prochymosin were carried according to the manual for the QuickChange site-directed mutagenesis kit.

Construction of an Expression Vector for Saccharomyces cerevisiae. A new expression vector was constructed by modification of the *S. cerevisiae* expression vector, pYES2. The glutathione-*S*-transferase (GST)[1] gene region of pGEX-4T-1 was amplified by PCR and inserted into *Hin*dIII and *Not*I sites of pYES2 to provide pYES2-GSTF with multicloning sites of *Bam*HI, *Eco*RI, and *Not*I derived from pGEX-4T-1 and *Xho*I, *Sph*I, and *Xba*I from pYES2. pGEM-T Easy vectors harboring cDNA inserts for human pepsinogen A and monkey prochymosin were digested with *Xho*I and *Xba*I, and the cDNAs were reinserted into the *Xho*I and *Xba*I sites in the pYES2-GSTF vector. The resulting plasmids, pYES2-GSTF-hPgA and pYES2-GSTF-mPgY, were transformed into *S. cerevisiae* strain INVSC1 according to the lithium acetate transformation protocol (*32*).

Expression and Purification of Mutant Pepsinogens and Prochymosins and Preparation of Active Enzymes. S. cerevisiae cells harboring pYES2-GSTF-pepsinogen/prochymosin plasmids were grown at 30 °C in shaker flasks in synthetic medium without uracil. At a cell density of A_{600} = 0.1, the cells were harvested and shifted to an induction medium, which contained 2% galactose. After induction for 4 days, the cultures were centrifuged to collect cells, which were then lysed at room temperature for 30 min with 5 volumes of Y-PER solution containing 5 mM 2-mercapto-

[1] Abbreviations: FGF, fibroblast growth factor; GST, glutathione-*S*-transferase; NT/NMN, neurotensin/neuromedin N precursor; POMC, proopiomelanocortin.

Table 1: Specific Activities of Human Pepsin A and Its Mutants against Typical Peptide Substrates

peptide	sequence and cleavage site[a]	human pepsin A and its mutants [nmol min^{-1} (μg protein)$^{-1}$][b]				
		wild type	$M^{289}D$	$L^{291}S$	$L^{298}Q$	$L^{291}S/L^{298}Q$
NT/NMN 142−151						
parent peptide	KIPYIL↓KRQL	0.93 ± 0.12	**1.0 ± 0.2**	**2.9 ± 0.3**	**2.8 ± 0.5**	**3.3 ± 0.3**
Glu^{148} variant	KIPYIL↓ERQL	2.4 ± 0.1	**1.8 ± 0.1**	**3.1 ± 0.7**	**3.2 ± 0.5**	**3.8 ± 0.5**
Phe^{148} variant	KIPYIL↓FRQL	10.2 ± 0.8	9.5 ± 2.0	**7.0 ± 1.0**	**4.0 ± 0.5**	**3.0 ± 0.3**
	KIPYILF↓RQL	3.3 ± 0.1	**10.2 ± 0.2**	**9.7 ± 0.1**	**11.2 ± 1.6**	**13.1 ± 1.3**
POMC 165−174						
parent peptide	AFPLEF↓KREL	0.86 ± 0.18	**2.5 ± 0.6**	**3.7 ± 0.9**	**3.7 ± 1.2**	**5.6 ± 1.2**
	AFPLE↓FKREL	2.0 ± 0.3	**0.54 ± 0.10**	**uc**[c]	**uc**	**uc**
Glu^{171} variant	AFPLEF↓EREL	1.5 ± 0.1	**1.9 ± 0.2**	**2.1 ± 0.1**	1.5 ± 0.1	**2.4 ± 0.2**
basic FGF 110−118[d]						
parent peptide	KYSSW↓YVAL	6.7 ± 0.4	6.7 ± 0.8	**4.9 ± 0.6**	6.6 ± 0.8	**3.6 ± 0.4**
Leu^{115} variant	KYSSWL↓VAL	9.7 ± 0.5	8.8 ± 1.9	**4.4 ± 1.7**	11.4 ± 1.0	**3.9 ± 0.7**
substance P						
parent peptide	RPKPQQF↓FGLM$_{NH2}$	4.9 ± 0.5	5.8 ± 0.8	**2.9 ± 0.6**	**2.1 ± 0.7**	**1.1 ± 0.1**
Lys^{8} variant	RPKPQQF↓KGLM	uc	**0.03 ± 0.01**	**0.02 ± 0.01**	**0.03 ± 0.01**	**0.06 ± 0.02**
dynorphin A 1−7[e]						
$Ala^{3}Phe^{7}$ variant	YGAF↓LRF	1.4 ± 0.1	**0.43 ± 0.05**	**0.73 ± 0.07**	**1.0 ± 0.1**	**0.28 ± 0.03**
$Ile^{3}Phe^{7}$ variant	YGIF↓LRF	0.72 ± 0.04	**0.36 ± 0.07**	**0.35 ± 0.04**	0.84 ± 0.13	**0.17 ± 0.02**
$Ile^{3}Lys^{5}Phe^{7}$ variant	YGIF↓KRF	0.06 ± 0.01	**0.02 ± 0.01**	**0.09 ± 0.01**	**0.18 ± 0.01**	**0.17 ± 0.01**

[a] All reactions were carried out in 0.2 M sodium formate buffer at pH 4.0. [b] All values were obtained by averaging determinations done 3 times and are given as mean ± SD. Values for mutants that were found to be significantly larger or smaller ($p < 0.05$) than those of the wild-type enzyme are shown in bold. [c] uc indicates that the peptide was uncleaved or the rate of its hydrolysis that gave a value less than 0.02 nmol min^{-1} (μg protein)$^{-1}$. [d] Lys^{115} variant (sequence = KYSSWKVAL) was not cleaved by the wild type and its mutants. [e] The sequence of the parent peptide is YGGFLRR.

ethanol and proteinase inhibitors (chymostatin, phosphoramidon, E-64, leupeptin, and antipain of 0.015, 0.2, 2, 0.1, and 0.4 mM, respectively). The Y-PER extract was mixed with an equal volume of 0.02 M sodium phosphate buffer, containing 0.5 M NaCl, and was applied to a column (0.7 inside diameter × 0.5 cm) of glutathione Sepharose 4B equilibrated with 0.01 M sodium phosphate buffer at pH 7.0, containing 0.25 M NaCl. The column was washed with the equilibration buffer and then with the 0.05 M Tris-HCl buffer at pH 8.0. The adsorbed GST fusion protein was eluted with 1 mL of 0.05 M Tris-HCl buffer at pH 8.0, containing 10 mM reduced glutathione, and the eluate was mixed with an equal volume of glycerol and stored at −20 °C.

Pepsin and chymosin were prepared by activation of GST-fusion pepsinogen, with the similar procedure to that used with various pepsinogens previously (*33*). Briefly, a solution of GST-fusion pepsinogen/prochymosin was mixed with $^1/_4$ volume of 0.15 N HCl, incubated at 14 °C for 1−5 h and mixed with 3 volumes of SP-Sephadex suspension (SP-Sephadex in 4 volumes of 0.05 M sodium acetate buffer at pH 5.0). The soluble fraction containing pepsin was then collected by centrifugation.

Molecular Modeling. The crystal structure of the complex between human pepsin A and a synthetic phosphonate inhibitor (*34*) was used as the initial model. Leu^{291} and Leu^{298} of the pepsin were replaced with Ser and Gln, respectively, and L-3-phenyllactic acid at P′1 of the inhibitor was substituted with 1-aminobutylic acid. Energy minimization was then carried out with the MOE program (Chemical Computing Group Inc., Quebec, Canada) to select the best model.

Statistics. All statistical analyses were performed using STATISTICA (Stat Soft. Inc.). Data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's *t* test.

RESULTS

Substrate Specificities of Pepsin A and Chymosin. The hydrolytic activities of human pepsin A and monkey chymosin against five types of peptides and their P′1 variants are summarized in Tables 1and 2. Each peptide was cleaved at one site, being identical between pepsin A and chymosin. An exception was Phe^{148}-neurotensin/neuromedin N precursor (NT/NMN) 142−151 and proopiomelanocortin (POMC) 165−174, which were cleaved at two sites. The residues at the P1 position were hydrophobic or aromatic in most cases. The optimal pH for the hydrolysis was 4 (Figure 1). The results distinctly show that the rates of cleavage differed significantly between two enzymes, depending on P′1 residues. Pepsins A cleaved the bonds where the P′1 position was occupied by hydrophobic and aromatic residues, such as Phe and Tyr. Because replacement of a hydrophobic/aromatic residue at P′1 by a charged one decreased the cleavage rate significantly in every type of peptide, it is apparent that peptides with charged residues such as Lys and Glu at P′1 were unfavorable substrates for pepsin A. Chymosin, in contrast, hydrolyzed peptides with charged residues as well as hydrophobic/aromatic residues at P′1 very rapidly. This showed that chymosin has diversified P′1 specificity accommodating various types of residues. Chymosin, however, was more specific on substrate types than pepsin A, showing NT/NMN 142−151, POMC 165−174, and their P′1 variants to be cleaved rapidly, while other types were cleaved very slowly.

Addressing Sites for Mutagenesis and Expression of Recombinant Enzymes. The S′1 subsites of pepsin A and chymosin were predicted to have significant roles in the different P′1 specificities between them. To date, the tertiary structures of human (*34*) and porcine (*16*, *19*, *35*) pepsins A and bovine chymosin (*20*, *36*) have been clarified, and the enzyme residues that possibly contact with the P′1 residue of a substrate have been anticipated (Figure 2). Although

Table 2: Specific Activities of Monkey Chymosin and Its Mutants against Typical Peptide Substrates

peptide	sequence and cleavage site[a]	monkey chymosin and its mutants [nmol min^{-1} (μg protein)$^{-1}$][b]			
		wild type	$D^{289}M$	$Q^{298}L$	$D^{289}M/Q^{298}L$
NT/NMN 142−151					
parent peptide	KIPYIL↓KRQL	15.0 ± 1.3	**12.5 ± 0.6**	**9.2 ± 1.6**	**7.4 ± 0.3**
Glu^{148} variant	KIPYIL↓ERQL	12.8 ± 1.8	**16.4 ± 1.3**	**8.2 ± 0.8**	11.0 ± 1.3
Phe^{148} variant	KIPYIL↓FRQL	21.4 ± 2.3	**32.9 ± 2.0**	**25.2 ± 1.8**	**61.5 ± 2.4**
	KIPYILF↓RQL	60.5 ± 2.8	**25.1 ± 1.5**	**36.4 ± 1.7**	**15.1 ± 1.0**
POMC 165−174					
parent peptide[c]	AFPLEF↓KREL	25.5 ± 1.7	**12.5 ± 1.5**	**12.8 ± 1.1**	**8.0 ± 0.6**
Glu^{171} variant	AFPLEF↓EREL	10.8 ± 1.7	**5.8 ± 0.5**	**5.8 ± 1.3**	**3.9 ± 0.4**
basic FGF 110−118					
parent peptide	KYSSW↓YVAL	0.17 ± 0.03	0.16 ± 0.01	0.16 ± 0.02	0.16 ± 0.01
Leu^{115} variant	KYSSWL↓VAL	0.03 ± 0.01	0.03 ± 0.01	**0.05 ± 0.01**	0.03 ± 0.01
Lys^{115} variant	KYSSW↓KVAL	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	**0.02 ± 0.01**
substance P					
parent peptide	RPKPQQF↓FGLM$_{NH2}$	0.15 ± 0.01	0.14 ± 0.02	0.15 ± 0.02	0.22 ± 0.08
Lys^{8} variant	RPKPQQF↓KGLM	0.20 ± 0.01	**0.04 ± 0.01**	**0.10 ± 0.01**	**uc**[d]
dynorphin A 1−7[e]					
$Ala^{3}Phe^{7}$ variant	YGAF↓LRF	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
$Ile^{3}Lys^{5}Phe^{7}$ variant	YGIF↓KRF	0.04 ± 0.01	0.06 ± 0.02	**uc**	**uc**

[a] All reactions were carried out in 0.2 M sodium formate buffer at pH 4.0. [b] All values were obtained by averaging determinations done 3 times and are given as mean ± SD. Values for mutants that were found to be significantly larger or smaller ($p < 0.05$) than those of the wild-type enzyme are shown in bold. [c] The Glu^{169}-Phe^{170} bond was not cleaved by the wild type and its mutants. [d] uc indicates that the peptide was uncleaved or the rate of its hydrolysis gave a value less than 0.02 nmol min^{-1} (μg of protein)$^{-1}$. [e] The sequence of the parent peptide is YGGFLRR. The $Ile^{3}Phe^{7}$ variant was not cleaved by the wild type and its mutants.

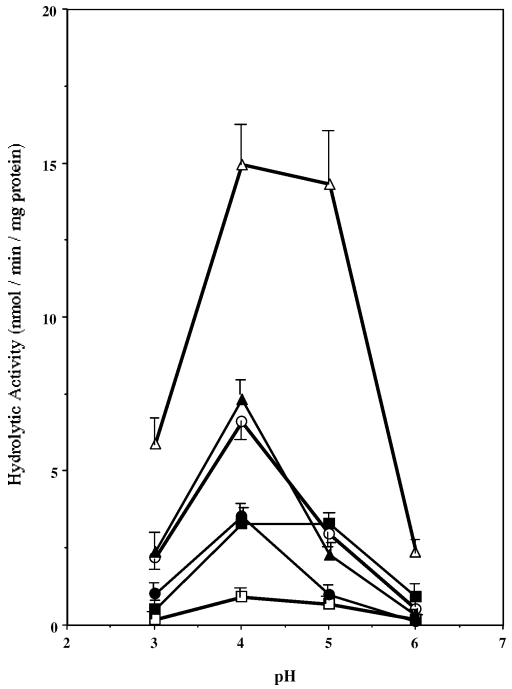

FIGURE 1: Dependence on pH of the hydrolysis of typical peptides. Hydrolytic activities of human pepsin A (○) and $L^{291}S/L^{298}Q$ double-mutated pepsin A (●) against basic FGF 110−118, human pepsin A (□) and $L^{291}S/L^{298}Q$ double-mutated pepsin A (■) against NT/NMN 142−151, and monkey chymosin (△) and $D^{289}M/Q^{298}L$ double-mutated chymosin (▲) against NT/NMN 142−151 are shown. The buffers used are sodium formate at pH 3−4 and sodium acetate at pH 5−6. Error bars indicate standard deviations ($n = 3$).

half of these residues such as Tyr^{189}, Ile^{213}, Ile^{300} are common between pepsin A and chymosin, the other half vary, particularly in the 289−299 loop region. Within the loop, although the region greatly differs among pepsins A and chymosins from various sources (Figure 3), the residues whose side chains possibly interact with P′1 are generally conserved or replaced by equivalent residues in pepsins A. The residues are Met^{289}, Leu/Ile/Val^{291}, Thr/Ser^{293}, and Leu^{298}. Although the loop region is more variable in chymosins and deletions of 4−6 residues make the S′1 pocket more open, the most frequent residues are Asp/Glu, Gln, and Gln at positions 289, 291, and 298, respectively. Thus, most residues in the loop are hydrophobic in pepsins A and hydrophilic in chymosins.

Differences in side-chain features of the loop residues were hypothesized to cause the different specificities. Thus, mutations in pepsin A were performed to replace specific residues with their chymosin-specific counterparts and vice versa. The resulting forms were $Met^{289}Asp$, $Leu^{291}Ser$, and $Leu^{298}Gln$ mutants and a $Leu^{291}Ser/Leu^{298}Gln$ double-residue mutant in human pepsin A and $Asp^{289}Met$ and $Gln^{298}Leu$ mutants and a $Asp^{289}Met/Gln^{298}Leu$ double-residue mutant in monkey chymosin. These were all expressed in yeast as GST fusion proteins. The amounts of expressed GST fusion pepsinogens, estimated by the hemoglobin-digestive assay (*37*), ranged from ∼0.2−1 mg/L culture. They were purified nearly homogeneously with GSH column chromatography, with the yield of about 30%. Each preparation contained a small amount of impurities, including yeast GST. Pepsins were released from the GST fusion pepsinogens by autocatalytic cleavage upon activation at pH 2 and freed from GST and activation peptides by SP-Sephadex treatment (Figure 4). The wild-type human pepsin A and monkey chymosin produced in yeast showed identical activities to the respective enzymes of stomach origin.

Change in the Substrate Specificity with Mutations. Significant differences in peptide hydrolysis were observed between the wild-type and mutant enzymes (Tables 1 and 2). In the case of pepsin A, significant changes in the cleavage rate were observed in every type of peptide. The rate of hydrolysis of peptides having hydrophobic/aromatic residues at P′1, such as substance P and basic fibroblast growth factor (FGF) 110−118, decreased with mutant enzymes, while that of peptides having charged residues at P′1, such as NT/NMN 142−151 increased (Table 1). Effects of mutation differed between positions 289, 291, and

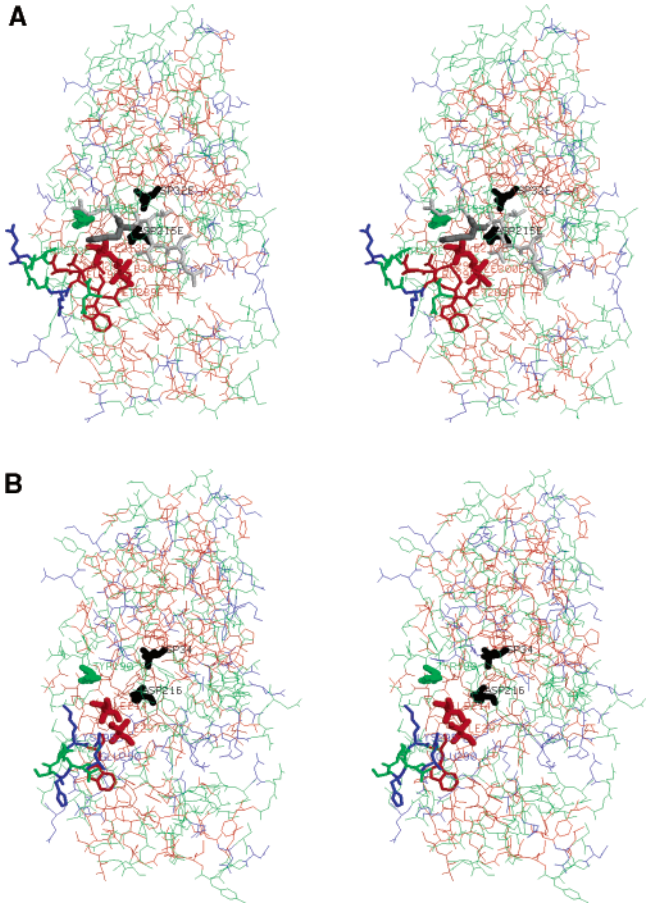

FIGURE 2: Stereoviews of the tertiary structures of human pepsin A (A) and bovine chymosin (B) highlighting the S′1 subsite and catalytic-site aspartates. The data were obtained from 3D structure databases at the National Center for Biotechnology Information [for human pepsin A, MMDB ID 12834 and PDB ID 1QRP, deposited by Fujinaga et al. in 1999 (*34*); for bovine chymosin, MMDB ID 3146 and PDB ID 4CMS, deposited by Newman et at. in 1991 (*20*)]. For human pepsin A, the structure of the complex between the enzyme and a synthetic phosphonate inhibitor is shown. The picture was generated using RASMOL version 2.5. Residues are shown with wireframes, distinguished by colors of red, green, and blue for nonpolar hydrophobic, polar but uncharged, and charged residues, respectively. Catalytic-site Asp^{32} and Asp^{215} (porcine pepsin-A numbering) are colored black, and inhibitor residues are indicated in gray. Tyr^{189}, Ile^{213}, and Ile^{300} in S′1 conserved in both pepsins and Asp^{32}, Asp^{215}, and L-3-phenyllactic acid at P′1 of the inhibitor are shown with thick wireframes. The residues in the 289−299 loop for S′1 are shown with medium-thickness wireframes. Catalytic aspartates and major S′1 residues are numbered, with suffix E in the case of pepsin A. Because of insertions/deletions, these numbers are marginally shifted between the two enzymes.

298, depending on the type of peptide. In NT/NMN and POMC peptides, the mutations at position 291 and 298 were most effective, whereas in dynorphin peptides, the mutation at position 289 produced the largest change in the cleavage rate. Effects of the single-residue mutation were amplified with the $Leu^{291}Ser/Leu^{298}Gln$ double-residue mutant. With this mutant, activities against peptides having a hydrophobic/aromatic residue at P′1 such as basic FGF 110−118, substance P, and $Ala^{3}Phe^{7}$-dynorphin A 1−7 decreased by factors of 1.4−5.0. On the contrary, activities against peptides having charged residues at P′1 such as NT/NMN 142−151 increased by factors of 1.6−6.5. In the cases of Phe^{148}-NT/NMN 142−151 and POMC 165−174, although two cleavage sites were found, one site (Leu^{147}-Phe^{148} and Glu^{169}-Phe^{170},

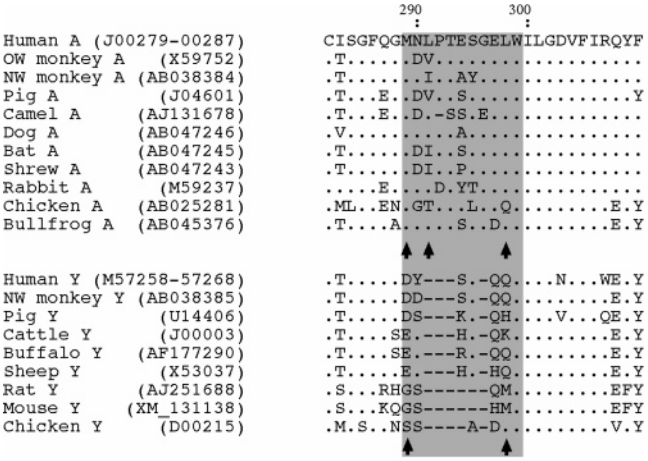

FIGURE 3: Comparison of the primary structures around the 289−299 loop regions of pepsins A and chymosins (denoted by Y) from various vertebrate sources. The porcine pepsin-A numbering is used. Dots indicate that amino acids that are identical to the human sequence. Deleted residues are shown by short bars. Residues in the 289−299 region are shaded. Arrow marks show residues that possibly contact the P′1 residue of a substrate and are targeted for mutation. Monkey sequences are those of the Japanese monkey (denoted by OW monkey) and the common marmoset (denoted by NW monkey). GenBank/EMBL/DDBJ accession numbers are given in parentheses. The human chymosin gene has been shown to be functionally inactive (*48*).

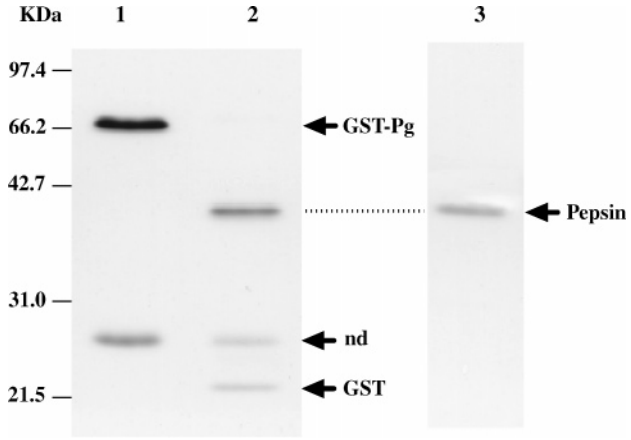

FIGURE 4: SDS−PAGE of GST fusion pepsinogen and isolated pepsin. PAGE was performed according to Laemmli (*49*), and the gel was stained with Coomasie Brilliant Blue. Results for wild-type human pepsin A are given. The positions of molecular mass markers are shown on the left side of the figure. Lane 1, GST fusion pepsinogen (GST-Pg) obtained by glutathione Sepharose 4B column chromatography; lane 2, GST fusion pepsinogen after glutathione Sepharose 4B affinity chromatography was incubated at pH 2.0 and 14 °C for 5 h. GST indicates GST released from the fusion protein, and nd indicates an impurity or yeast GST; lane 3, pepsin fraction after SP-Sephadex treatment. The NH_2-terminal sequence of pepsin was confirmed to be identical to that reported previously by us (*29*).

respectively) were preferred by pepsin A and the other (Phe^{148}-Arg^{149} and Phe^{170}-Lys^{171}, respectively) by chymosin. It became clear that pepsin-A mutants cleaved predominantly chymosin-preferring sites. These results show that the hydrolytic specificities of the mutants were changed to chymosin-type specificity, most obviously in the double-residue mutant.

Mutant chymosins were examined with the same peptide set (Table 2). Significant changes in activity were observed in NT/NMN 142−151, POMC 165−174, and their variants.

Table 3: Kinetic Constants for the Hydrolysis of Typical Peptides by Human Pepsin A and Its Mutants[a]

	human pepsin A and its mutants				
peptide	wild type	Met[289]Asp	Leu[291]Ser	Leu[298]Gln	L[291]S/L[298]Q
NT/NMN 142−151					
parent peptide					
K_m	123 ± 10	82 ± 12	90 ± 10	116 ± 17	82 ± 4
k_{cat}	4.2 ± 0.7	3.1 ± 0.2	7.6 ± 0.6	10 ± 2	11 ± 2
k_{cat}/K_m	0.037 ± 0.003	0.038 ± 0.006	**0.085 ± 0.003**	**0.089 ± 0.026**	**0.13 ± 0.02**
Glu[148] variant					
K_m	68 ± 2	84 ± 27	62 ± 3	85 ± 2	64 ± 11
k_{cat}	5.5 ± 0.4	4.9 ± 1.0	6.1 ± 0.1	9.4 ± 1.23	7.7 ± 0.2
k_{cat}/K_m	0.081 ± 0.004	**0.061 ± 0.008**	**0.098 ± 0.004**	**0.11 ± 0.02**	**0.13 ± 0.02**
Phe[148] variant (L[147]-F[148] cleavage)					
K_m	17 ± 3	22 ± 2	42 ± 8	53 ± 12	95 ± 26
k_{cat}	12 ± 1	12 ± 1	15 ± 2	16 ± 2	13 ± 2
k_{cat}/K_m	0.72 ± 0.05	**0.54 ± 0.04**	**0.354 ± 0.03**	**0.29 ± 0.03**	**0.14 ± 0.03**
Phe[148] variant (F[148]-R[149] cleavage)					
K_m	15 ± 2	14 ± 3	26 ± 3	28 ± 6	38 ± 10
k_{cat}	4.4 ± 0.2	12 ± 1	20 ± 1	34 ± 3	32 ± 3
k_{cat}/K_m	0.29 ± 0.03	**0.85 ± 0.10**	**0.77 ± 0.04**	**1.2 ± 0.2**	**0.84 ± 0.18**
POMC 165−174					
parent peptide					
K_m	69 ± 4	233 ± 52	246 ± 24	139 ± 14	188 ± 13
k_{cat}	1.4 ± 0.2	13 ± 2	20 ± 4	12 ± 1	21 ± 2
k_{cat}/K_m	0.021 ± 0.001	**0.058 ± 0.006**	**0.081 ± 0.007**	**0.086 ± 0.005**	**0.11 ± 0.01**
Glu[171] variant					
K_m	304 ± 33	90 ± 12	360 ± 75	411 ± 89	463 ± 93
k_{cat}	8.9 ± 2.0	7.9 ± 1.6	23 ± 3	26 ± 5	30 ± 6
k_{cat}/K_m	0.029 ± 0.002	**0.086 ± 0.006**	**0.066 ± 0.005**	**0.063 ± 0.005**	**0.065 ± 0.005**
basic FGF 110−118					
K_m	70 ± 8	104 ± 18	105 ± 16	324 ± 55	184 ± 38
k_{cat}	15 ± 2	23 ± 4	17 ± 2	61 ± 6	21 ± 4
k_{cat}/K_m	0.22 ± 0.02	0.22 ± 0.01	**0.16 ± 0.01**	0.19 ± 0.02	**0.12 ± 0.01**
substance P					
K_m	54 ± 9	139 ± 14	73 ± 8	357 ± 45	238 ± 30
k_{cat}	8.0 ± 1.7	23 ± 2	6.4 ± 1.1	30 ± 5	6.5 ± 1.0
k_{cat}/K_m	0.15 ± 0.01	0.17 ± 0.02	**0.087 ± 0.006**	**0.085 ± 0.005**	**0.027 ± 0.002**

[a] All reactions were carried out in 0.2 M sodium formate buffer at pH 4.0. The units of K_m, k_{cat}, and k_{cat}/K_m are μM, S^{-1}, and μM^{-1} S^{-1}, respectively. All values were obtained by averaging determinations done 3 times and are given as mean ± SD. k_{cat}/K_m values for mutants that were found to be significantly larger or smaller ($p < 0.05$) than those of the wild-type enzymes are shown in bold.

Activities against NT/NMN 142−151, its Phe[148] variant (cleavage of the Phe[148]-Arg[149] bond), POMC 165−174 (cleavage of the Phe[170]-Lys[171] bond), and its Glu[171] variant were decreased, maximally by a factor of 4.0 with the Asp[289]-Met/Gln[298]Leu double-residue mutant. The results showed charged residues such as Lys and Glu at P′1 to be less accommodated than with the wild-type, except that the Asp[289]Met mutant occasionally accommodated Glu at P′1 more effectively than the wild-type enzyme. On the contrary, the rate of hydrolysis of Phe[148]-NT/NMN 142−151 (cleavage of the Leu[147]-Phe[148] bond) increased maximally by a factor of 2.9. In contrast to the NT/NMN and POMC peptides, the rates of hydrolysis of basic FGF 110−118, dynorphin A, substance P, and their variants were less affected by mutation. Because these types of peptides were much less accommodated in wild-type chymosin than in NT/NMN and POMC peptides, their access to the active site might be limited, resulting in the lack of apparent effects of mutation. These results indicate that, although chymosin was specific on substrate types, its hydrolytic specificity was changed to pepsin-type specificity.

A comparison of Michaelis−Menten kinetics with typical peptides provide k_{cat}/K_m values in line with the rates for hydrolysis, as shown in Tables 3 and 4. In pepsin-A mutants, the improved hydrolysis of peptides having a charged residue at P′1 was found to be mainly due to an increase in the k_{cat} value. Typically, in the double-residue mutant of pepsin A, the k_{cat} value for the hydrolysis of POMC 165−174 was augmented by a factor of 15, while the K_m value changed by a factor of 2.7. In contrast, a decrease in the cleavage rate of a hydrophobic/aromatic-residue combination by mutant pepsins was mainly associated with an increase in the K_m value; for instance, the cleavage rate of substance P with the Leu[291]Ser/Leu[298]Gln double-residue mutant increased by a factor of 4.4, whereas the k_{cat} value changed by a factor of 0.81. In chymosin mutants, the alternation in Michaelis−Menten parameters was the reverse to the pepsin-A mutants in most cases. A decrease in the rates of hydrolysis of POMC 165−174 and NT/NMN 142−151 was mainly due to a decrease in the k_{cat}. Typically, the k_{cat} value for POMC 165−174 hydrolysis by the Asp[289]Met/Gln[298]Leu double-residue mutant was lowered by a factor of 5.1 and the K_m value, only by a factor of 1.5.

DISCUSSION

Human pepsin A and monkey chymosin showed different hydrolytic specificities, depending on P′1 residues in the substrates. Pepsins A reported so far have essentially similar specificities, hydrolyzing peptide bonds connecting hydrophobic or aromatic residues (*8*, *12*). Although chymosin is

Table 4: Kinetic Constants for the Hydrolysis of Typical Peptides by Monkey Chymosin and Its Mutants[a]

peptide	monkey chymosin and its mutants			
	wild type	$Asp^{289}Met$	$Gln^{298}Leu$	$D^{289}M/Q^{298}L$
NT/NMN 142−151				
parent peptide				
K_m	59 ± 5	72 ± 21	95 ± 5	87 ± 12
k_{cat}	41 ± 8	35 ± 2	30 ± 2	27 ± 3
k_{cat}/K_m	0.70 ± 0.07	**0.52 ± 0.12**	**0.32 ± 0.02**	**0.31 ± 0.04**
Glu^{148} variant				
K_m	111 ± 3	54 ± 5	62 ± 7	46 ± 10
k_{cat}	46 ± 2	32 ± 2	17 ± 1	22 ± 3
k_{cat}/K_m	0.42 ± 0.02	**0.60 ± 0.03**	**0.27 ± 0.02**	**0.50 ± 0.06**
Phe^{148} variant				
(L^{147}-F^{148} cleavage)				
K_m	83 ± 9	54 ± 11	29 ± 4	16 ± 2
k_{cat}	28 ± 3	39 ± 3	12 ± 1	16 ± 1
k_{cat}/K_m	0.33 ± 0.03	**0.71 ± 0.09**	**0.40 ± 0.05**	**1.0 ± 0.1**
(F^{147}-R^{148} cleavage)				
K_m	105 ± 20	28 ± 5	33 ± 5	24 ± 4
k_{cat}	191 ± 26	42 ± 5	49 ± 2	17 ± 1
k_{cat}/K_m	1.8 ± 0.1	**1.5 ± 0.1**	**1.5 ± 0.2**	**0.70 ± 0.08**
POMC 165−174				
parent peptide				
K_m	375 ± 42	288 ± 25	229 ± 41	250 ± 33
k_{cat}	219 ± 24	101 ± 15	90 ± 14	43 ± 6
k_{cat}/K_m	0.59 ± 0.02	**0.35 ± 0.02**	**0.39 ± 0.02**	**0.17 ± 0.01**
Glu^{171} variant				
K_m	304 ± 66	65 ± 4	463 ± 93	114 ± 16
k_{cat}	63 ± 13	14 ± 1	52 ± 8	12 ± 1
k_{cat}/K_m	0.21 ± 0.02	0.23 ± 0.02	**0.11 ± 0.01**	**0.10 ± 0.01**

[a] All reactions were carried out in 0.2 M sodium formate buffer at pH 4.0. The units of K_m, k_{cat}, and k_{cat}/K_m are μM, S^{-1}, and μM^{-1} S^{-1}, respectively. All values were obtained by averaging determinations done 3 times and are given as mean ± SD. k_{cat}/K_m values for mutants that were found to be significantly larger or smaller ($p < 0.05$) than those of the wild-type enzymes are shown in bold.

more substrate-specific than pepsin A and this might cause low general proteolytic activity of chymosin (*6*), the present study demonstrates for the first time that chymosin accommodates charged residues such as Lys and Glu, as well as hydrophobic ones, at P′1. Although bovine chymosin is known to have significant activity against combinations of hydrophobic residues such as the Phe^{105}-Met^{106} bond of κ-casein of bovine milk, causing milk clotting (*38*, *39*), previous reports have documented that peptides released from α_{s1}-casein and β-casein by the action of bovine chymosin often feature Lys, Glu, or Asp at their N termini (*40*−*42*). This shows the cleavage sites to be those expected from S′1 subsite specificity.

The S′1 subsites of pepsins are unique in that they have group-specific characteristics. X-ray structural and kinetic studies have revealed a series of specificity pocket S4−S′3 subsites on either side of the catalytic aspartate (*16*−*20*, *36*). The S1 subsite of pepsins is thought to be the primary determinant of specificity because the constituent residues are mainly hydrophobic and aromatic in all types and the cleavage probability is much higher here than at any other site (*12*). The S′1 subsite, in contrast, varies between pepsins and features two types of residues, i.e., conservative and group-specific residues. Conservative residues Tyr^{189}, Ile^{213}, and Ile^{300} are common to most pepsins. The group-specific residues are located in the 289−299 loop, including Met^{289}, Leu/Val^{291}, Thr^{293}, and Leu^{298} in human/porcine pepsin A and Asp/Glu^{289}, Ser/His^{295}, and Gln/Lys^{298} in monkey/bovine chymosin. Because the conservative residues are hydrophobic or aromatic in nature, the S′1 subsite of a pepsin family has potential to accommodate a hydrophobic P′1 residue. Thus, the group-specific residues are thought to be important to modify the S′1 subsite, resulting in a unique S′1 specificity of an individual pepsin group. In pepsin A, the overall hydrophobic character of the S′1 subsite is responsible for their preference for hydrophobic residues. Thus, the combination of hydrophobic residues accommodated at the P1−P′1 positions has been clarified by Fruton (*8*) as supported by the present work. The group-specific residues are charged or polar residues in chymosins, in contrast. This makes the S′1 subsite of chymosin consistent with conservative hydrophobic and group-specific polar/charged residues. Such dual characteristics in S′1 has not been observed in other types of pepsins, including gastricsin and pepsin B, and might result in the diversified S′1 specificity of chymosin to accommodate multiple types of residues at P′1.

Although it is necessary to identify the residues responsible for S′1 specificity, mutagenesis of the S′1 subsite has hitherto not been performed, with the S1 subsite attracting most attention. Putative residues in S1 are Ile^{30}, Asp^{32}, Tyr^{75}, Thr^{77}, Phe^{111}, Leu^{112}, Phe^{117}, Ile^{120}, and Gly^{217} in porcine pepsin A and conserved or equivalent residues in other pepsins. Residues targeted for mutation include Asp^{32}, Thr^{77}, and Gly^{76} in porcine pepsin A (*21*, *23*, *25*, *26*) and Tyr^{75}, Val^{110}, Phe^{111}, and Ala^{115} in bovine chymosin (*22*, *24*, *43*). The replacements generally lowered the catalytic efficiency or changed the specificity. Residues in other subsites including Ser^{35} in S′2, Thr^{218}, Thr^{222}, and Glu^{287} in S2 (*44*), Ser^{219} in S3 of porcine pepsin A (*45*), and Thr^{218} and Lys^{220} in S2 of bovine chymosin (*22*, *46*, *47*) have also been targeted and thereby shown to be important for catalytic efficiency. The present study demonstrates that the S′1 subsite specificities of pepsin A and chymosin could be successfully altered by replacement of group-specific residues in the 289−299 loop.

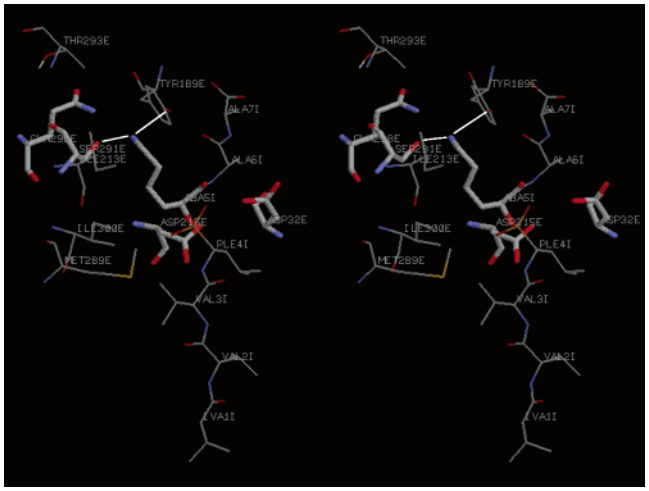

FIGURE 5: Stereoview of the S′1 subsites of the Leu[291]Ser/Leu[298]Gln double-residue mutant of human pepsin A in complex with a phosphonate inhibitor. The structure was built with the MOE program using a pepsin−inhibitor complex (*34*) as the initial model. Phenyl radical of L-3-phenyllactic acid of the original inhibitor was replaced with 1-aminopropyl radical, resulting in the introduction of an aliphatic chain having an ϵ-amino group at P′1 of the inhibitor. Selected residues of mutant pepsin including catalytic-site aspartates and residues in the S′1 subsite are given. Residues are numbered with suffixes E and I to those of the pepsin and the inhibitor, respectively. Residues are shown with wireframes, distinguished by colors of gray, red, light blue, and yellow for carbon, oxygen, nitrogen, sulfur, and phosphorus atoms, respectively. Asp[32], Asp[215], Ser[291], Gln[298], and the P′1 residue of the inhibitor (abbreviated to ABA5) are shown with thick wireframes. White lines indicate hydrogen bonds. The picture was created using RASMOL version 2.5.

The fact that substrates containing charged residues such as Lys and Glu at P′1 were efficiently hydrolyzed by pepsin-A mutants showed that the normal S′1 specificity was altered to the chymosin type. Asp, Ser, and Gln at the positions 289, 291, and 298 in the mutants in place of Met, Leu, and Leu, respectively, in the wild type, thus play an important role in the recognition of charged residues such as Lys at P′1.

Preference for charged residues is thought to be caused by the increase in hydrogen-bonding interactions. The best-fitting model for the Leu[291]Ser/Leu[298]Gln double-residue mutant of pepsin A in complex with an inhibitor having a Lys side chain at P′1 shows that a most probable hydrogen bond to the ϵ-NH_2 group at P′1 is donated by the hydroxyl group of Ser[291], supporting the present hypothesis (Figure 5). When the side chain at P′1 is replaced with another charged or polar group, hydrogen bonds might be donated by the carbonyl O of Gln[298] as well as the hydroxyl group of Ser[291]. The hydroxyl group of Tyr[189], which is a conservative P′1 residue, might also be involved in a hydrogen-bonding network between the P′1 side chain and S′1 subsite residues (*17*). Although the major subsites of pepsin including S1 have the extensive hydrophobic character to accommodate hydrophobic/aromatic residues, hydrogen-bonding interactions have also been shown to be essential for substrate binding (*17*). For instance, Thr[77], Thr[222], and Glu[287] in S2 have been anticipated as hydrogen donors to substrates (*17*, *19*), and this was evidenced by site-directed mutagenesis (*23*, *25*, *44*). The present study shows that the introduction of polar residues to the S′1 subsite of pepsin changed the substrate-binding specificity significantly, because of a new hydrogen-bonding network. Leu[291]Ser and Leu[298]Gln mutants demonstrated a significant increase in catalytic efficiency (k_{cat}/K_m), mainly because of an increase in k_{cat}; therefore, their cleavage of the P1−P′1 bond proceeded rapidly, probably because of a decrease in the free energy of the transition-state stabilization of the enzyme−substrate complex. Although Glu at P′1 was accepted by the mutants, the increase in the catalytic efficiency was less pronounced than with peptides having Lys at P′1. This might indicate that the hydrogen-bonding networks from the side chains were weak. Moreover, in the case of the Met[289]Asp mutant, the catalytic efficiency was occasionally decreased, possibly because of electrostatic repulsion between Glu at P′1 and the Asp[298]. The fact that peptides having hydrophobic residues at P′1 such as substance P and basic FGF 110−118 were less effectively hydrolyzed by pepsin-A mutants than the wild type is clearly due to the replacement of the hydrophobic residues in S′1 by hydrophilic alternatives. Here, the K_m values appear to be of primary relevance. With mutant chymosins, the results were reverse to those in pepsin-A mutants, with the hydrolytic activity of Asp[289]Met and Gln[298]Leu mutants toward peptides having Lys at P′1 decreasing significantly, mainly because of a decrease in the k_{cat} values.

In conclusion, pepsin A has been clarified to have a strict preference for hydrophobic/aromatic residues at P′1, while chymosin showed a diversified preference accommodating charged residues as well as hydrophobic/aromatic ones. Residues 289, 291, and 298 of pepsin A and residues 289 and 298 of chymosin are found to be involved in the determination of their unique P′1 specificities.

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