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New World Monkey Pepsinogens A and C, and Prochymosins. Purification, Characterization of Enzymatic Properties, cDNA Cloning, and Molecular Evolution[1]

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Pepsinogens A and C, and prochymosin were purified from four species of adult New World monkeys, namely, common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), squirrel monkey (*Saimiri sciureus*), and capuchin monkey (*Cebus apella*). The occurrence of prochymosin was quite unique since this zymogen is known to be neonate-specific and, in primates, it has been thought that the prochymosin gene is not functional. No multiple form has been detected for any type of pepsinogen except that two pepsinogen-A isozymogens were identified in capuchin monkey. Pepsins A and C, and chymosin hydrolyzed hemoglobin optimally at pH 2–2.5 with maximal activities of about 20, 30, and 15 units/mg protein. Pepsins A were inhibited in the presence of an equimolar amount of pepstatin, and chymosins and pepsins C needed 5- and 100-fold molar excesses of pepstatin for complete inhibition, respectively. Hydrolysis of insulin B chain occurred first at the Leu15-Tyr16 bond in the case of pepsins A and chymosins, and at either the Leu15-Tyr16 or Tyr16-Leu17 bond in the case of pepsins C. The presence of different types of pepsins might be advantageous to New World monkeys for the efficient digestion of a variety of foods. Molecular cloning of cDNAs for three types of pepsinogens from common marmoset was achieved. A phylogenetic tree of pepsinogens based on the nucleotide sequence showed that common marmoset diverged from the ancestral primate about 40 million years ago.

Key words: molecular evolution, New World monkeys, pepsinogen A, pepsinogen C, prochymosin.

Pepsinogens are zymogens of pepsins, the major proteolytic enzymes in vertebrate gastric juices. They have been purified from the gastric mucosa of various animals and the presence of four major groups has been demonstrated (*1–3*). They are pepsinogen A and pepsinogen C (progastricsin) in adult animals, and prochymosin and pepsinogen F in neonates. These groups have been shown to have diverged from a common ancestor during the evolution of vertebrates (*3, 4*). The relative levels of these pepsinogens in the gastric mucosa differ among mammals. Type-A pepsinogens are nearly the only components of rabbit (*5*) and Asiatic black bear (*6*) pepsinogens, and they are predominant in the stomachs of man (*7*), Japanese monkey (*8, 9*), cow (*10*), pig (*11*), and house musk shrew (*12*). In macaque monkeys, pepsinogens A are known to account for nearly 90% of the total pepsinogens. By contrast, type-C pepsinogens are the exclusive components of rodent pepsinogens (*13, 14*). Prochymosins are expressed predominantly in the neonatal gastric mucosa of artiodactyls (*15, 16*) and carnivores (*17*), but they have not been demonstrated in primates including man (*18*) and Japanese monkey (*19*). The human prochymosin gene has been shown to be inactivated by mutations (*18*). Pepsinogen F is a neonate-specific pepsinogen that has been molecular-cloned in rabbit (*20*) and rat (*3*). Isozymogens are occasionally observed for each type of pepsinogen, especially pepsinogen A (*1, 21*). The occurrence of multiple pepsinogens may be correlated to the gastric digestion of a variety of foods.

The order primates has been divided into suborders Prosimii and Anthropoidea. The latter are divided further into platyrrhines (New World monkeys) and catarrhines (Old World monkeys and hominoids). To date, primate pepsinogens have only been investigated in man and Old World monkeys such as Japanese monkey, information about New World monkey pepsinogens not being available. Since man (*21*) and Old World monkeys (*9*) are known to have several pepsinogen isozymogens and New World monkeys have been shown to have diverged from catarrhines nearly halfway through primate evolution (*22, 23*), the study of New World monkey pepsinogens is necessary to clarify the origin of the multiplicity of primate pepsinogens. Moreover, since some New World monkeys such as common marmoset eat animal as well as plant food (*24*), it would be interesting to determine whether or not New World monkey pepsins have adapted for the digestion of a variety of foods.

In the present study, we undertook the purification and

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characterization of pepsinogens and pepsins of four species of New World monkeys. The results show the occurrence of type-A and C pepsinogens, and prochymosin in adult New World monkey stomachs. The occurrence of prochymosin was quite unique since this zymogen is known to be neonate-specific. Multiplicity of these three types of pepsinogens has scarcely been found. The enzymatic properties of these three types of pepsins are clarified. cDNA cloning of the three types of pepsinogens is achieved for common marmoset, and structural and molecular evolutional analyses are carried out.

MATERIALS AND METHODS

Chemicals—DEAE-Sephacel and the Mono Q column (HR 5/5) were purchased from Pharmacia LKB Biotech., Uppsala, Sweden; bovine hemoglobin (substrate powder) was from Worthington Diagnostic Systems, Freehold, NJ; pepstatin was from the Peptide Institute, Minoh; and reagents for amino acid analysis were from Wako Pure Chemicals, Tokyo. The mRNaid kit was obtained from BIO101, Vista, CA; the SuperScript™ choice system for cDNA synthesis and cloning vector pUC18 were from Life Technologies, Rockville, MD; cloning vector λgt10 and the DNA *in vitro* packaging kit (Gigapack Gold) were from Stratagene Cloning System, La Jolla, CA; the Thermo Sequenase™ cycle sequencing kit was from Amersham Life Science, Cleveland, OH; and restriction endonucleases and DNA-modifying enzymes were from Toyobo, Osaka. All other chemicals were of reagent or analytical grade.

Monkeys—Four species of New World monkeys were used, namely, common marmoset (*Callithrix jacchus*), cotton-top tamarin (*Saguinus oedipus*), squirrel monkey (*Saimiri sciureus*), and capuchin monkey (*Cebus apella*). Stomachs were removed from adult monkeys immediately after sacrifice by exsanguination *via* bilateral carotid arteries under deep anesthesia with ketamine hydrochloride and sodium pentobarbital, in accordance with the guidelines of the Primate Research Institute, Kyoto University.

Assaying of Proteolytic Activity—Potential pepsin activity of pepsinogen, as well as pepsin activity, was determined at pH 2.0 and 37°C with a solution of approximately 2% (w/v) hemoglobin as the substrate (*25*). One unit of enzymatic activity was defined as the amount that gave an increase of 1.0 in the absorbance at 280 nm per min under the assay conditions. For examination of the substrate specificity, the enzyme was assayed with various protein substrates by the same procedure. A turbidimetric milk-clotting assay was performed at pH 5.5, with 0.1% casein as the substrate, by the method of McPhie (*26*). The hydrolysis of oxidized insulin B chain was carried out as described previously (*27*).

Purification of Pepsinogens—The procedures for the purification of pepsinogens from the four monkey species were essentially the same as those used in the case of Japanese monkey (*8, 28*) and house musk shrew (*12*). In brief, several pepsinogen isozymogens in a crude homogenate of gastric mucosa were resolved by column chromatography on DEAE-Sephacel. Different types of pepsinogens and their isozymogens were finally purified by FPLC on a Mono Q column, HR 5/5. Examination of the purity and determination of the molecular mass were carried out by SDS-PAGE (*29*). Pepsinogens were activated to the respective pepsins according to the method of Kageyama and Takahashi (*30*) to characterize some enzymatic properties.

Determination of Protein Concentrations—The protein concentrations in the solutions of the enzymes at each step of the purification or in the purified state were determined by measuring the absorbance at 280 nm, applying the molar extinction coefficient calculated from the amino acid composition and the molecular mass.

Amino Acid Analysis—Samples for amino acid analysis were hydrolyzed under HCl vapor at 150°C for 1 h with a Waters PICO-TAG™ Work Station (Millipore, MA). The amino acids were analyzed with an amino acid analyzer (model 835; Hitachi, Tokyo).

Amino Acid Sequence Determination—The NH_2-terminal amino acid sequence was determined using an automatic protein sequencer (model 477A; Applied Biosystems, Foster City, CA, USA).

Isolation and Characterization of the cDNA Clones—Total gastric RNA was isolated, extracted and purified by the guanidinium thiocyanate/cesium chloride centrifugation method. Poly(A)$^+$ RNA was purified with an mRNaid kit. Double-stranded complementary DNA was prepared by the procedure of Gubler and Hoffman (*31*) using a cDNA-synthesis kit. After methylation of the internal *Eco*RI sites and addition of *Eco*RI linkers, the cDNA was fractionated according to size by agarose-gel electrophoresis. The cDNA

TABLE I **Pepsinogen levels in gastric mucosae of four species of New World monkeys.** One individual was used for each species except that two individuals were used in the case of squirrel monkey. Since the sex and ages of the two squirrel monkeys were the same, the average values are given. The levels in those of Japanese monkey and man are shown for comparison

Species	Age (yr)	Sex	Body wt (kg)	Stomach wt. (g)	Mucosal wt (g)	Mucosal protein (mg)	Pepsinogen level (units)	Specific activity (units/mg protein)	Relative levels in terms of activity of pepsinogens A and C, and prochymosin (%)			Ref
									A	C	Y	
Callithrix jacchus (common marmoset)	5.5	M	0.38	2.5	1.1	68	320	4.7	45	48	7	This study
Cebus apella (capuchin monkey)	13	F	2.1	12	4.6	54	290	5.4	62	24	14	"
Saimiri sciureus (squirrel monkey)	13	M	0.75	4.5	1.6	120	490	4.1	43	37	20	"
Saguinus oedipus (cotton-top tamarin)	3	M	0.57	3.0	1.1	64	182	2.8	41	43	16	"
Macaca fuscata (Japanese monkey)	3	M	4.9	21	–	276	1,834	6.6	88	12	nd	(*9*)
Homo sapiens[a] (man)	–	–	–	100	40	1,357	2,028	1.5	73	24	nd	(*7*)

[a]Residual 3% of the activity was due to procathepsin E. –, not determined. nd, non-detectable

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that ranged from 1 to 3 kb in length was ligated into the *Eco*RI site of λgt10. The phages were packaged, and the recombinants were selected by plating on *Escherichia coli* strain C600Hfl. Nitrocellulose filters that carried denatured recombinant DNA were screened by plaque hybridization with ^{32}P-labeled cDNAs for Japanese monkey pepsinogens A and C (*19*), and rat prochymosin (*3*). The *Eco*RI inserts of the hybridizing phages were cloned into pUC18 plasmids. The sequence was determined by the dideoxy chain-termination method (*32*) using a Thermo Sequenase™ cycle sequencing kit and a DNA sequencer (model 4200S-1; LI-COR Inc., Lincoln, NE).

RESULTS

Purification of Pepsinogens—The total pepsinogen levels in the gastric mucosa of New World monkeys were in the range of 2.8–5.4 units/mg protein in terms of relative specific activity (Table I). Pepsinogens in crude homogenates of the gastric mucosa of the New World monkeys were separated into 2–3 fractions by DEAE-Sephacel chromatography at pH 7.0 (Fig. 1). Three types of pepsinogens, namely, pepsinogens A and C, and prochymosin, were finally purified by successive FPLC. The relative levels of the three types of pepsinogens in each monkey are summarized in Table I. No isozymogen was found for any type of pepsinogen except that two pepsinogen-A isozymogens were separated on FPLC for capuchin monkey. Each purified pepsinogen was found to be homogeneous on SDS-PAGE (Fig. 2). The apparent molecular masses of the pepsinogens were different. Prochymosins, pepsinogens A, and pepsinogens C exhibited values of about 42, 40, and 38 kDa, respectively.

The amino acid compositions of the respective types of pepsinogens, namely pepsinogens A and C, and prochymosins, quite resembled each other in the four monkey species, although remarkable differences were obvious between the different types of pepsinogens (Table II). Distinct differences were found in the ratios of Glx/Asx and Leu/Ile. The values were notably higher in pepsinogens C than pepsinogens A. In the case of prochymosins, the Glx/Asx ratio was close to those of pepsinogens A, and the Leu/Ile ratio was intermediate between those of pepsinogens A and C. These differences are known to be characteristic of these three types of pepsinogens from different animal sources such as Japanese monkey (*19*), as shown in Table II for comparison. The contents of basic residues such as Lys and Arg were higher in prochymosins than the other two types of pepsinogens, this being consistent with the observation that prochymosins were eluted in earlier fractions on DEAE-Sephacel chromatography. The NH_2-terminal 10-res-

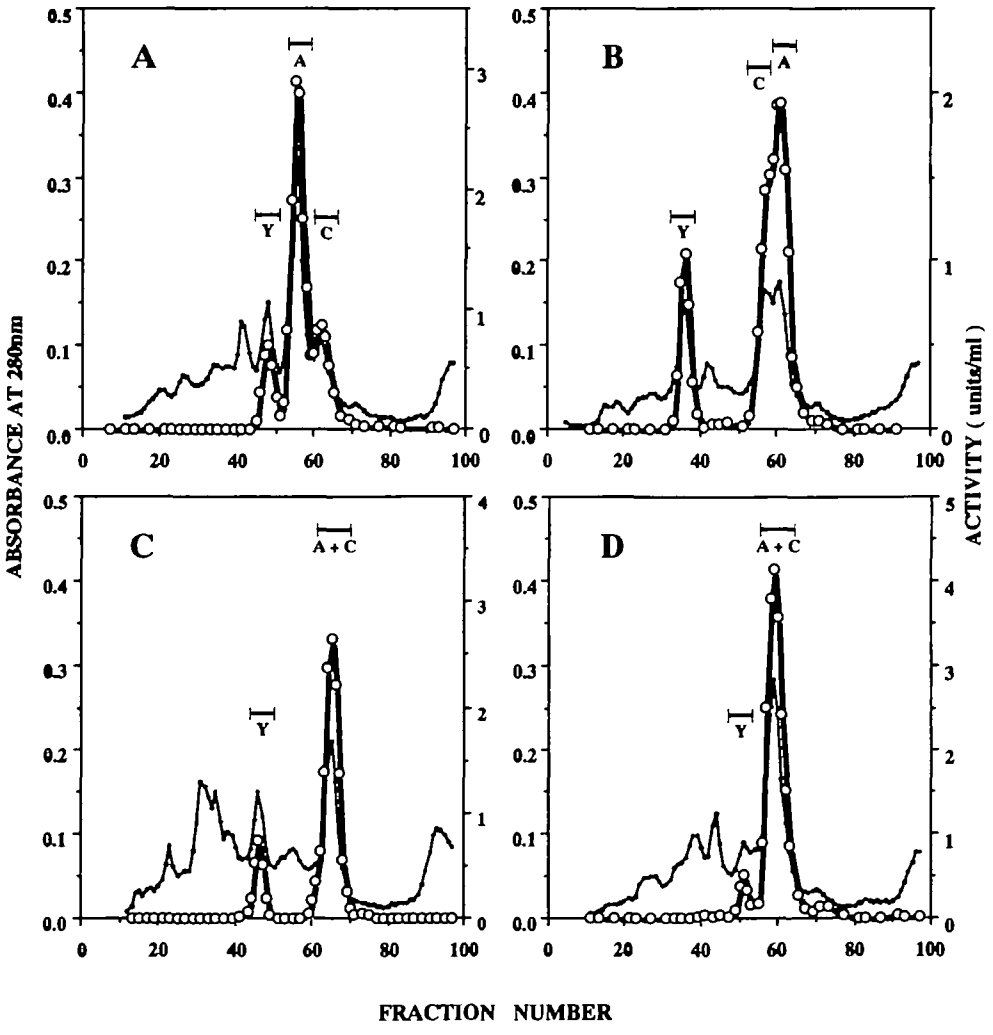

Fig 1 **Chromatography of crude homogenate supernatants of gastric mucosae of New World monkeys on a column (1.5 × 30 cm) of DEAE-Sephacel.** The column was equilibrated with 0.01 M sodium phosphate buffer, pH 7 0, and adsorbed proteins were eluted with a 1-liter linear gradient of NaCl, from 0 to 0 5 M, in the same buffer The fraction size was 10 ml. ○, hemoglobin-digestive activity; ●, absorbance at 280 nm. The fractions under the bars were pooled separately. A, C, and Y stand for pepsinogens A and C, and prochymosin, respectively. The NaCl gradient starts from fraction number 1 (A) Capuchin monkey (*Cebus apella*), (B) squirrel monkey (*Saimiri sciureus*), (C) cotton-top tamarin (*Saguinus oedipus*), and (D) common marmoset (*Callithrix jacchus*)

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idue sequences of common marmoset pepsinogens A and C, and prochymosin were determined to be LYKVSLIKKK, TVVKVPLKKF, and SGIVRIPLHK, respectively. The three types of pepsinogens were greatly different in their NH_2-terminal sequences.

Enzymatic Properties of Pepsins—Since the enzymatic properties of the respective types of pepsins were essentially the same in the four species of New World monkeys, the results for common marmoset pepsins are shown in the figures as typical examples.

Digestive activities toward protein substrates: New World monkey pepsins were active at low pH values, similarly to those from other animal sources. They exhibited general proteolytic activities toward various protein substrates. Bovine hemoglobin was the best substrate, followed by bovine serum albumin and bovine casein. Bovine γ-globulin was hydrolyzed by each pepsin much less efficiently The optimal pHs for hemoglobin digestion were around 2.0 for pepsins A and C, and 2.5 for chymosins (Fig. 3A). The maximal activities were highest for pepsins C, followed by for pepsins A and chymosins. The relative extents of hydrolysis of albumin and casein by marmoset pepsin A at pH 2.0 were 72 and 54%, respectively, of hemoglobin hydrolysis. These values were 13 and 75% in the case of pepsin C, and 34 and 55% in that of chymosin.

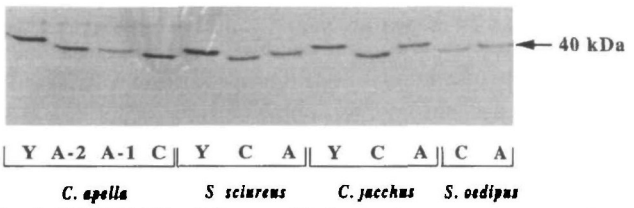

Fig 2 **SDS-PAGE of the purified pepsinogens.** An amount corresponding to approximately 1 μg protein was loaded on each lane. Protein was stained with Coomassie Brilliant Blue. A, C, and Y stand for pepsinogens A and C, and prochymosin, respectively Monkey species *C. apella*, capuchin monkey, *S sciureus*, squirrel monkey, *C jacchus*, common marmoset, *S oedipus*, cotton-top tamarin

Hydrolytic specificity for insulin B chain: Several sites were cleaved by the New World monkey pepsins (Fig. 4). The most susceptible site(s) to hydrolysis was the Leu15-Tyr16 bond in the case of pepsins A and chymosins, and the Leu15-Tyr16 and Tyr16-Leu17 bonds in the case of pepsins C. Secondary cleavage by pepsins A and C occurred at the Phe25-Tyr26 and Phe24-Phe25 bonds, respectively. Whilst, secondary cleavage by chymosins was not specified since the rate was too slow.

Milk-clotting activity: The relative activities to that of porcine pepsin A were determined for the three types of pepsins of New World monkeys. They were nearly 80, 50, and less than 5% for chymosins, pepsins A, and pepsins C, respectively.

Inhibition by pepstatin: The New World monkey pepsins were inhibited by pepstatin (Fig. 3B). The susceptibilities to pepstatin differed between the pepsins. Pepsins A were inhibited almost completely in the presence of over an equimolar amount of pepstatin, while chymosins and pepsins C needed about 5- and 100-fold molar excesses of pepstatin for complete inhibition, respectively.

Molecular Cloning of cDNAs—Among 1,500 recombinant clones of λgt10 prepared from the gastric mucosa of adult common marmoset, three types of clones were screened by hybridization with the radiolabeled cDNA probes for Japanese monkey pepsinogens A and C, and rat prochymosin.

TABLE II **Amino acid compositions of pepsinogens A (A) and C (C), and prochymosin (Y) of common marmoset.** The compositions of Japanese monkey pepsinogens A and C (*19*), and bovine prochymosin (*45*) are shown for comparison. Each value is based on the amino acid sequence deduced from the nucleotide sequence.

Amino acid	Number of residues per molecule of protein					
	Common marmoset			Japanese monkey		Bovine
	A	C	Y	A	C	Y
Asp	18	13	26	23	13	22
Asn	22	16	12	17	17	15
Thr	25	26	23	26	27	25
Ser	43	42	39	46	39	35
Glu	14	18	14	14	18	14
Gln	19	25	22	19	26	25
Pro	19	19	16	21	19	16
Gly	36	37	33	36	37	32
Ala	26	21	16	20	22	16
Cys	6	6	7	6	6	6
Val	23	24	31	27	26	26
Met	7	8	8	4	7	8
Ile	30	16	18	29	16	22
Leu	28	33	28	30	33	29
Tyr	20	21	18	18	23	22
Phe	17	23	19	17	21	19
Lys	8	9	11	7	8	15
His	2	3	9	3	3	6
Arg	4	6	11	5	5	8
Trp	5	6	4	5	6	4
Total	372	372	365	373	372	365
Mr	39,860	40,623	40,190	39,950	40,591	40,505
Glx/Asx	0.83	1.48	0.95	0 83	1.47	1.05
Leu/Ile	0 93	2 06	1.56	1 03	2.06	1.32

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The inserted DNA fragments were subcloned into the pUC18 plasmid and definitively identified on sequence analysis since the NH_2-terminal sequences of marmoset pepsinogens had been determined at the protein level. The cDNA clones for pepsinogens A and C, and prochymosin were obtained (Figs. 5–7). The deduced amino acid sequence of these pepsinogens consisted of three regions, *i.e.*, a pre-peptide (signal peptide), a pro-peptide (activation segment), and the active enzyme. The amino acid sequences of primate pepsinogens were compared (Fig. 8). Although the similarities between marmoset pepsinogens A and C, and prochymosin ranged from 45 to 56%, they showed high similarities with the respective pepsinogens of man, Japanese monkey, and rhesus monkey (values of similarity between these primates were 85–87, 89–93, and 83% in the cases of pepsinogens A and C, and prochymosin, respec-

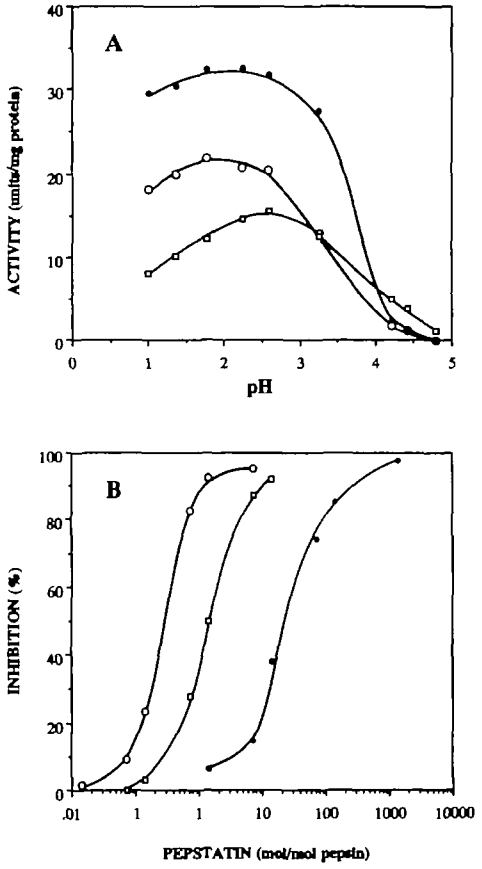

Fig. 3. **(A) pH-dependent hemoglobin-digestive activity of common marmoset pepsin A (○), pepsin C (●), and chymosin (□). (B) The inhibitory effects of pepstatin on the respective enzymes.**

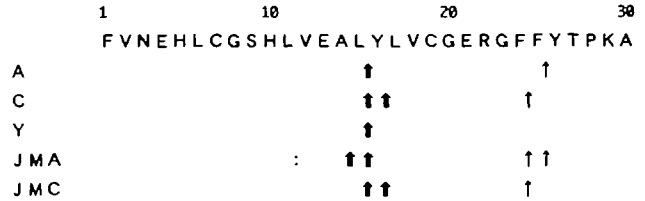

Fig. 4. **Cleavage sites of oxidized insulin B chain for pepsins A (A) and C (C), and chymosin (Y) from common marmoset.** The results for Japanese monkey pepsins A (JMA) and C (JMC) are shown for comparison. Primary (↑), secondary (↑), and other minor (·) cleavage sites are shown under the sequence.

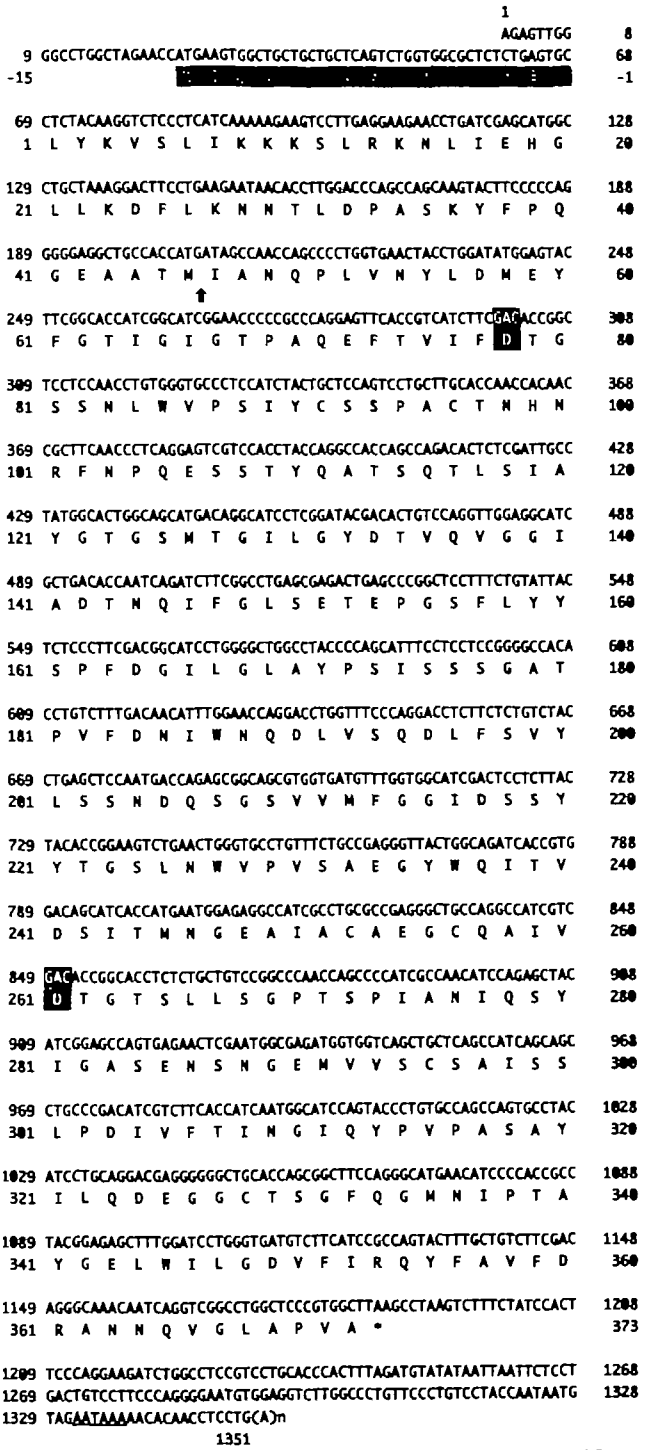

Fig 5 **The nucleotide sequence and deduced amino-acid sequence of the cDNA (pNW794) for common marmoset pepsinogen A.** The two active-site Asp residues are shown in filled boxes. The potential signal sequence is shown by shading. The putative cleavage site for release of the activation segment from pepsinogen is indicated by an arrow. The position of the polyadenylation signal sequence, AATAAA, in the 3′-untranslated region is underlined.

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tively). The positions of the insertions/deletions were the same in these primate pepsinogens except that one more deletion was found in the NH_2-terminus of marmoset pepsinogen A.

Molecular Evolution—The nucleotide sequences of the open reading frames of the cDNAs for marmoset pepsinogens A and C, and prochymosin, and of the cDNAs for pepsinogens from other animals were compared. Maximum-likelihood analyses of DNA sequences were performed with the PUZZLE v. 4.0.2 program (*33*) using the HKY model of sequence evolution (*34*). The constructed phylogenetic tree is shown in Fig. 9. The tree shows clearly the four clusters of pepsinogens, namely, clusters of pepsinogens A and C, pepsinogen F, and prochymosin. Pepsinogen C seems to

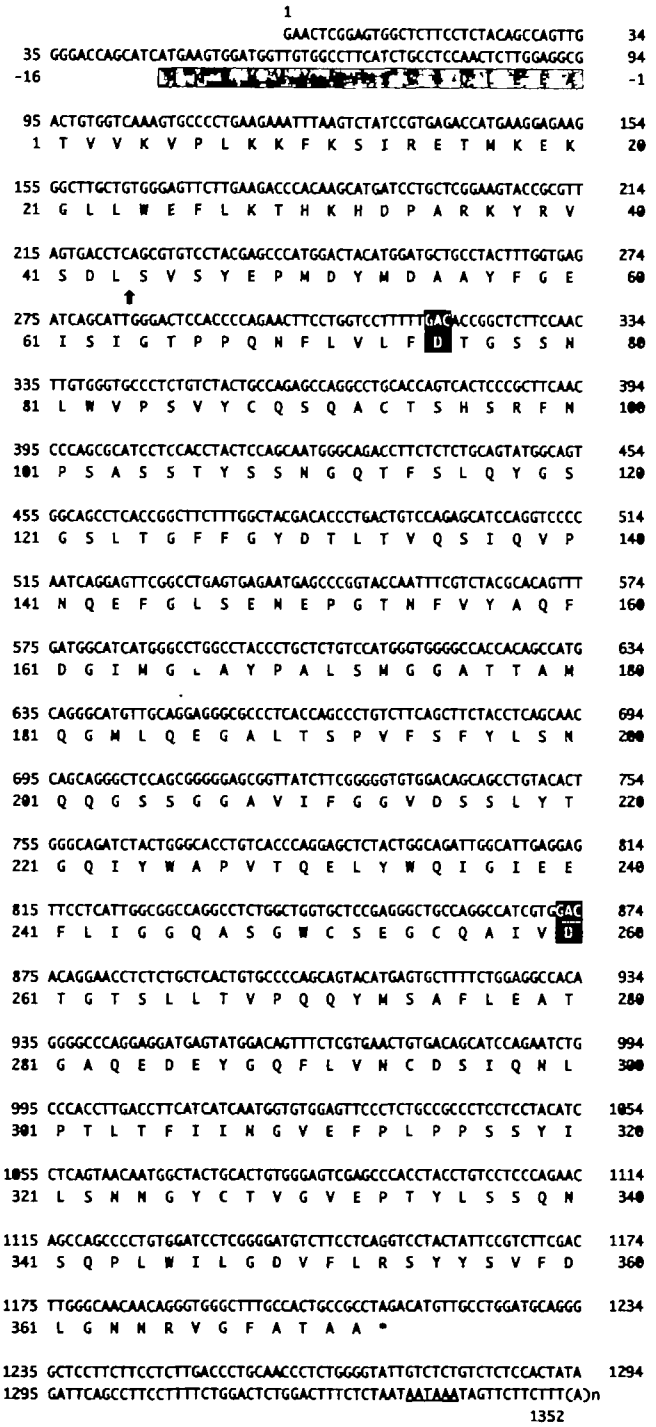

Fig. 6 **The nucleotide sequence and deduced amino-acid sequence of the cDNA (pNW791) for common marmoset pepsinogen C.** Modification of the figure was carried out as described in the legend to Fig 5.

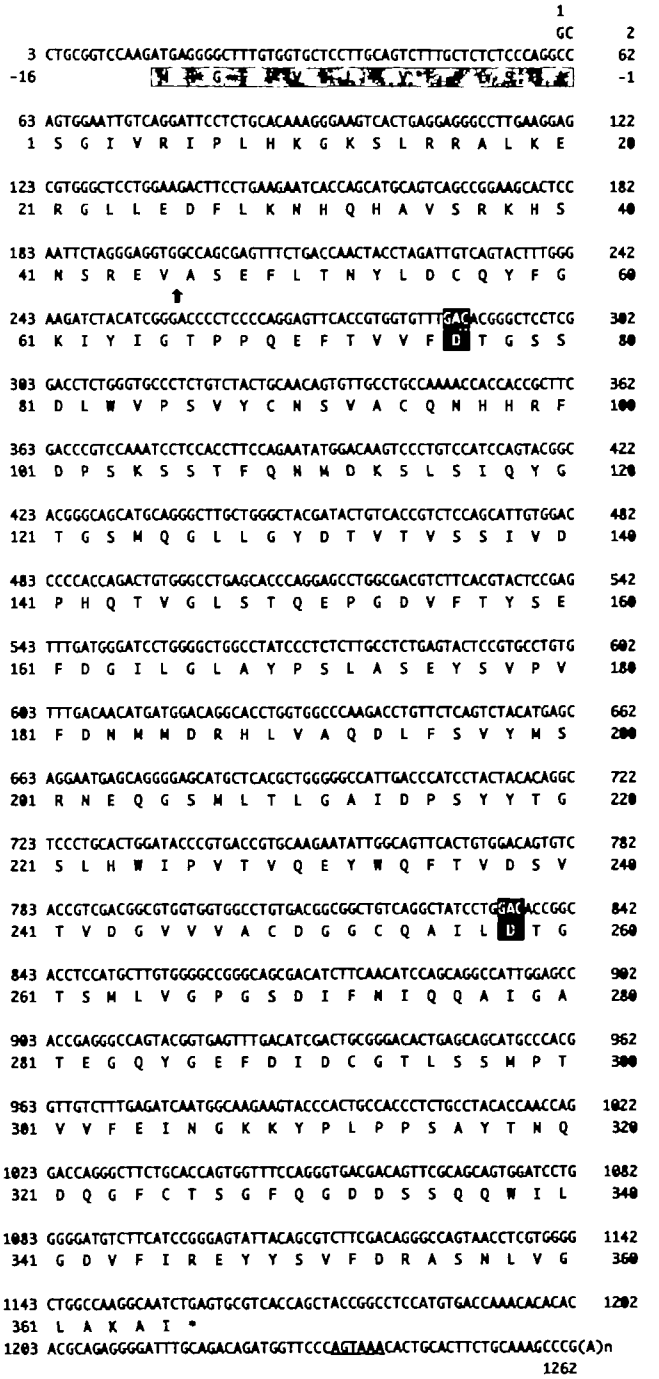

Fig 7 **The nucleotide sequence and deduced amino-acid sequence of the cDNA (pNW813) for common marmoset prochymosin.** Modification of the figure was carried out as described in the legend to Fig 5.

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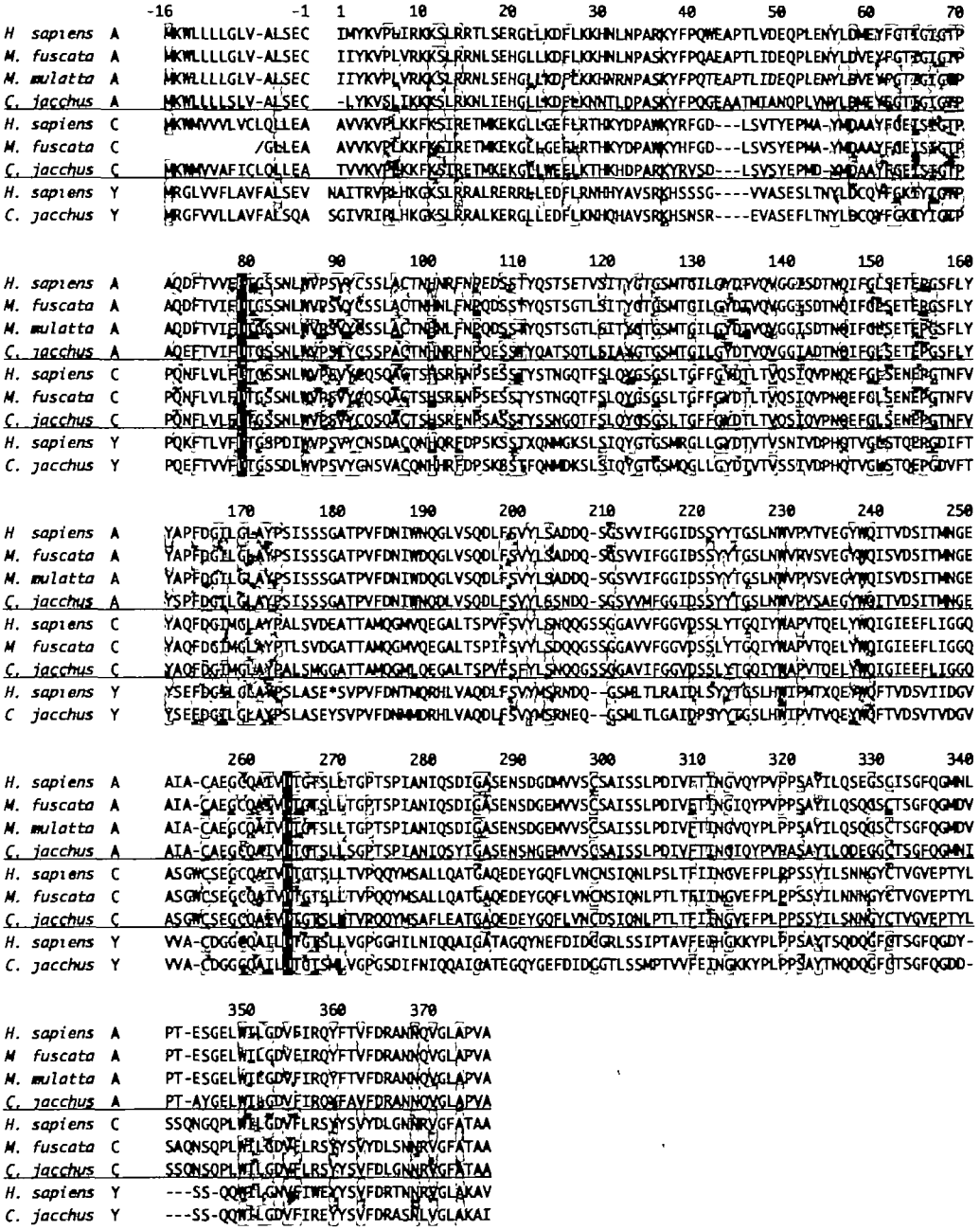

Fig. 8. **Comparison of the amino acid sequences of common marmoset (*Callithrix jacchus*) pepsinogens A and C, and prochymosin with those of other primate pepsinogens.** A, C, and Y stand for pepsinogens A, C, and prochymosin, respectively. The amino acid sequences were deduced from the cDNA or genomic DNA sequences of human (*Homo sapiens*) pepsinogens A (*41*) and C (*42*), and prochymosin (*18*), Japanese monkey (*Macaca fuscata*) pepsinogens A and C (*19*), and rhesus monkey (*Macaca mulatta*) pepsinogen A (*46*) The numbering is based on the sequence of Japanese monkey pepsinogen A. Bars represent deleted residues. Common residues in these pepsinogens are shown by shading. The two active-site Asp residues are shown in filled boxes. The human prochymosin gene has been shown to be functionally inactive due to the occurrence of stop codon and frame shift deletion mutations (*18*). These mutated positions are indicated by an asterisk (position at 179) and X (positions at 111 and 234), respectively.

have diverged at an earlier time than the others, followed by prochymosin. From the branching lengths of the phylogenetic tree, the divergence time for the marmoset branching point is calculated to be about 40 million years ago, when we use a date of 100 million years ago for the divergence time of extant mammalian orders (*35*).

DISCUSSION

Three types of pepsinogens, namely, pepsinogens A and C, and prochymosin, were purified from the gastric mucosa of adult New World monkeys. The occurrence of prochymosin in adult monkeys is quite remarkable since this zymogen is known to be expressed specifically in fetuses and neonates

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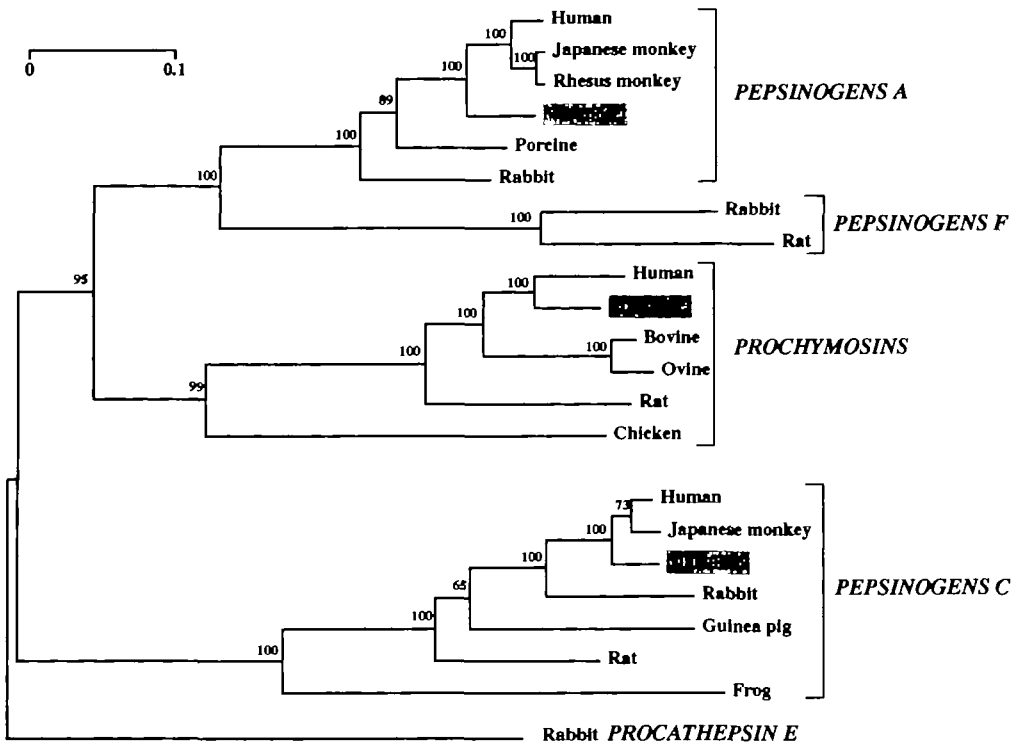

Fig. 9. **Maximum likelihood tree for vertebrate pepsinogens A and C, prochymosin, and pepsinogen F based on aligned protein-coding nucleotide sequences.** The cDNA sequences other than those for marmoset pepsinogens are those of pepsinogens A from man (*41*), Japanese monkey (*19*), rhesus monkey (*46*), pig (*47, 48*), and rabbit (*20*), pepsinogens F from rabbit (*20*) and rat (*3*), prochymosins from man (*18*), cow (*45*), sheep (*49*), rat (*3*), and chicken (*50*), and pepsinogens C from man (*42*), Japanese monkey (*19*), rabbit (*3*), guinea pig (*14*), rat (*51*), and frog (*52*). In the case of the Japanese monkey and rabbit pepsinogens A, cDNA for the major component was used for tree construction. Rabbit procathepsin E (*53*) was used as an outgroup. The branch lengths are proportional to the evolutionary distances, which are given as the average numbers of the substitution per site. The scale is shown in the upper part of the figure The numbers beside nodes represent the bootstrap values obtained for 1,000 replicates. Marmoset pepsinogens are shaded.

(*16, 36*). Although prochymosin has not been found in primates such as Japanese monkey (*19*) or man (*18*), and the human prochymosin gene has been shown to be inactive due to mutational changes (*18*), the present results showed that the prochymosin gene is functional in New World monkeys, even at the adult stage. It is known that chymosins from various animal sources have different proteolytic activities, showing that the enzyme has species-specific characteristics (*16*). The zymogen might play significant roles in gastric digestion in New World monkeys judging from the high general proteolytic activity of its active form, chymosin. Since some New World monkeys such as marmoset are known to be carnivorous (insectivorous) as well as herbivorous (*24*), the occurrence of prochymosin might be favorable for monkeys to digest a variety of foods efficiently.

Pepsinogens A and C were the major components and their relative levels were largely similar in each New World monkey. To date, pepsinogens A and C have been investigated in various animals (*1, 2*), their relative levels being very different between animal species. In Old World monkeys such as Japanese monkey, the levels of pepsinogens A have been shown to be much higher than those of pepsinogens C, accounting for 80–90% of the total (*9*). Since the levels of pepsinogens A and C were largely similar in New World monkeys, it is suggested that the expressional mechanisms for the genes for pepsinogens A and C are different between New World monkeys and Old World monkeys.

Isozymogen was not found in any type of pepsinogen of New World monkeys, except that two pepsinogens A were identified for capuchin monkey. Multiple forms of both type-A and C pepsinogens are known to occur in various mammals including Japanese monkey (*9, 19*) and man (*21*). The multiplicity is quite extreme in type-A pepsinogens. Although some of these multiple forms are generated through post-translational modifications such as phosphorylation (*8*) and glycosylation (*10, 37*), most of them are known to be the products of different genes. At least four pepsinogen-A genes have been demonstrated in man (*38*), and three different pepsinogen-A cDNAs have been obtained in Japanese monkey (*19*). Whilst, when we assume that the capuchin monkey pepsinogen-A isozymogens might be the products of allelic genes, since the generation of a isozymogen through modifications such as phosphorylation or glycosylation might result in a significant change in the chromatographic behavior or molecular mass (*8, 37*), it is likely that the duplication of the gene for each type of pepsinogen did not occur in New World monkeys. These results suggest that the duplication of the pepsinogen-A gene occurred in Old World monkeys and man independently, or in their common ancestor after the divergence from New World monkeys.

Some enzymatic properties of pepsins A and C, and chymosin of New World monkeys were examined. The hemoglobin-digestive activities of New World monkey pepsins A and C were largely similar to those of the respective pepsins of Japanese monkey (*8*). Although the activities of New World monkey chymosins were slightly lower than those of pepsins A and C, they were higher than those of chymosins from other mammalian sources which have been reported to be in the range of 3–50% of those of porcine pepsin A (*16*)

The hydrolytic specificity for insulin B chain showed that pepsins prefer hydrophobic and aromatic amino acids at the P1 and P'1 positions, as has been demonstrated for

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other mammalian pepsins (*1*, *39*). The sites susceptible to hydrolysis are largely common between different types of pepsins. However, some differences were obvious. Pepsins A have been shown to first cleave either the Leu15-Tyr16 or Ala14-Leu15 bond (*27*). The ratios of the cleavage of these bonds have been determined to be 1:0.6 and 1:0.25 for porcine and Japanese monkey pepsins A, respectively (*27*). For New World monkey pepsins A, however, the primary cleavage of Ala14-Leu15 bond was scarcely observed. This suggests that the active site of New World monkey pepsin A strictly prefers the Leu15-Tyr16 bond. New World monkey pepsin C first cleaved either the Leu15-Tyr16 or Tyr16-Leu17 bond, this being different from in the case of pepsin A. This is common to Japanese monkey pepsin C. The preference for hydrolysis of the Tyr16-Leu17 bond as well as the Leu15-Tyr16 bond is thought to be characteristic of type-C pepsins. New World monkey chymosin cleaved the Leu15-Tyr16 bond highly predominantly compared to the other bonds. This specificity resembles that of pepsin A. Since bovine chymosin has been shown to rapidly cleave the Leu17-Val18 and Glu13-Ala14 bonds (*15*), the differences between monkey and cow might reflect that chymosins have species-specific characteristics, this being consistent with that the hemoglobin-digestive activities of chymosins are greatly different between animals (*16*).

The difference in the susceptibility to pepstatin was clear between the three types of pepsins. To date, susceptibility to pepstatin has been known to be quite different between pepsins A and C from various animal sources, and this difference is useful for distinguishing these two types of pepsins (*8*, *27*). The intermediate susceptibility of chymosin has only been reported for the bovine enzyme (*40*). The present results showed that such susceptibility might be common to chymosins from different sources.

Successful cloning of cDNAs for the three types of common marmoset pepsinogens enabled us to analyze the structures of New World monkey pepsinogens and the evolutionary relationships between primates. The estimated date of divergence of New World monkeys from the ancestral primate is 40 million years ago. This agrees well with the fossil record (*22*) and dates estimated from other molecular markers (*23*). As reported previously (*4*, *14*, *20*), pepsinogen is thought to a useful molecular marker for clarifying the evolution of mammalian orders and families. The structural features of marmoset pepsinogens A and C were similar to those of pepsinogens A and C of Japanese monkey (*19*) and man (*41*, *42*), respectively. Regarding prochymosin, we found a noticeable structure. Chymosins are known to be optimally active in the weakly acidic pH region around pH 3–4, whilst pepsins A and C are active in a more acidic pH region such as pH 2 (*1*). The basic residue at position 269 in Fig. 8 (Lys in the case of bovine chymosin) has been thought to facilitate hydrolysis of a substrate at a weakly acidic pH and to be characteristic of chymosins (*43*, *44*). Since marmoset chymosin has Met at this position, a hydrophobic residue common to pepsins A and C, the shift of the pH optimum to a higher pH would not be expected. This assumption was supported by the results of a characterization study showing that marmoset chymosin is optimally active at pH 2.5, *i.e.* much lower than the optimal pH (3.5–4) of bovine chymosin (*16*, *44*).

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