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The Complete Amino Acid Sequence of Newborn Dog Prochymosin

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강아지 프로카이모신의 전 아미노산 서열

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요 약

생후 2주일 되는 강아지의 위에서 카이모신을 추출하고 이온교환 크로마토그래피로 정제하였다. 카이모신 아미노산 서열의 반은 아미노산 서열 분석법으로, 또 프로카이모신의 전아미노산 서열은 프로카이모신 cDNA의 염기서열로부터 결정하였다. 강아지 프로카이모신의 아미노산 서열은 송아지와는 79%, 돼지 펩신 노진 A와는 54%의 상동성을 보였다. 프로펩티드의 크기와 활성효소의 N-말단 아미노산 잔기의 위치는 다른 프로카이모신과 같았다. 강아지 카이모신의 pH 3.2에서 단백질 분해활성의 최대값은 돼지 펩신의 pH 2에서 값의 3~4% 밖에 되지 않았으나, 응유활성은 송아지보다 훨씬 높았다. 강아지의 위 추출물에 대한 pH 5.2에서의 한천 겔 전기이동으로 프로카이모신과 카이모신에는 두 가지의 현저한 유전적 변이형이 존재함을 확인하였다. 두 변이형은 아미노산 조성, N-말단 서열, 그리고 효소성질에서 차이가 없었다. 송아지와 강아지 카이모신의 기질결합에 관여하는 아미노산 잔기는 다음과 같이 서로 달랐다(돼지 펩신의 서열번호로 표시함) : Ser12 Thr (S_4), Leu30 Val (S_1/S_3), His74 Gln (S'_2), Val111 Ile (S_1/S_3), Lys220 Met (S_4). 강아지 카이모신의 단백질 분해활성이 낮은 것은 송아지의 Asp 303이 강아지에서는 Val303으로 바뀐 때문이라고 생각된다.

Key words: newborn dog prochymosin, newborn dog chymosin cDNA, amino acid sequence of newborn dog chymosin, aspartic proteinase.

I. INTRODUCTION

Calf chymosin is the milk clotting enzyme in traditional cheese rennet, and during the past 150 years numerous papers on the properties of this aspartic proteinase have been published[5,7,8]. For many years it was assumed that the chymosins were characteristic for young ruminants. By immunological methods, however, we observed that an antiserum to calf chymosin could precipitate a protein from extracts of newborn dog gastric mucosa as well as from extracts of gastric mucosa from other species of young mammals[9].

The present studies are the first detailed study of a nonruminant chymosin and the purpose was dual; first to study the developmental biology of canine gastric proteases and second to prepare newborn dog chymosin in amounts that were sufficient for analyses of enzymatic properties and determination of the amino acid sequence. Consequently each stomach was extracted separately and the contents of chymosin and pepsin were detemined by immunological methods[24]. The extracts were then pooled for preparation of pure chymosin as described in this paper.

Determination of the amino-terminal amino acid sequence showed that newborn dog and calf chymosin possessed 78~83% amino acid sequence identity but showed considerable differences in their enzymatic properties. When tested against canine milk, the clotting activity of newborn dog chymosin is about 6 to 8 times greater than that of calf chymosin. Thus, nature has provided us with two closely related enzymes which have significant differences in specificities and activities. A detailed analysis of their structures may therefore contribute to an understanding of the significance of the amino acid residues that are responsible for the differences in these properties. This prompted us to determine the amino acid sequence of pup prochymosin and chymosin.

II. MATERIALS AND METHODS

1. Chemicals

Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, USA) and Takara (Otsu, Japan). Trypsin and chymotrypsin were from Wako Pure Chemicals (Osaka, Japan). *Staphylococcus aureus* V8 protease, iodoacetamide, dithiothreitol (DTT), *o*-iodosobenzoic acid, CNBr, trishydroxymethyl-aminomethane (TRIS), N-tris (hydroxymethyl)methylglycine (tricine), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 2-(2-nitrophenylsulfenyl)-3-bromo-3-methylindolenine (BNPS-skatole) and 3-mercaptopropionic acid were from Sigma (St Louis, MO, USA). Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), ammonium peroxidisulfate and N,N,N′,N′-tetramethylethylenediamine were from Bio Rad (Richmond, USA). Trifluoroacetic acid (TFA) and sequencer reagents were from Applied Biosystems (Foster, USA). Polyvinyldifluoride (PVDF) membranes were from Millipore (Boston, USA). Agar (Noble) was from Gibco (Detroit, USA). DE 32 ion exchanger was from Whatman (Maidstone, UK). All other chemicals and reagents were of the purest commercial grade available.

2. Purification of newborn dog chymosin

Stomachs were obtained from dogs that died during their first two weeks of life. The stomachs were excised and opened less than 5 hrs after death, flushed with water and stored

at −20℃ until use.

In order to investigate the protease content of individual stomachs, each stomach was extracted separately with water in an ice-cooled homogenizer. The tissue debris was removed by centrifugation(12,000g for 15 min at 4℃), and was extracted once more under the same conditions.

For preparative purification of chymosin 2ℓ of mixed extracts were obtained from 24 stomachs (total weight 240 g). With continuous stirring 0.35 M aluminum sulfate was added until a pH of 4.0 was obtained; the pH was then immediately raised to 6.0 by addition of 0.5 M disodium phosphate. The gelatinous precipitate was removed by centrifugation as above. After dialysis against distilled water, the extract was applied to a column of DEAE-cellulose(30cm×2.5cm) equilibrated in 0.05 M sodium phosphate, pH 6.0. Most non-enzymic proteins did not bind to the column under these conditions. After washing of the column until A_{280} of the effluent was less than 0.05, elution of bound proteins took place with a linear gradient from 0.05 to 0.45 M sodium phosphate, pH 5.5 (total gradient volume 2ℓ). Throughout the purification, fractions were monitored for contents of chymosin by zymograms and rocket immunoelectrophoresis[11]. The elution pattern was essentially the same as for bovine chymosin and prochymosin[4], with prochymosin being eluted from 0.15 to 0.2 M sodium phosphate and chymosin being eluted from 0.25 to 0.4 M sodium phosphate.

Chymosin was purified to homogeneity from pooled fractions, or from selected peak fractions by ion exchange chromatography on Mono Q media. The columns were eluted with a linear gradient from 0.05 to 0.55 M ammonium acetate, pH 5.4. Two homogeneous, chromatographically distinct, main components were obtained[7].

The fractions containing prochymosin were pooled, dialyzed against 0.2% pyridine, concentrated by pressure dialysis and subjected to gel filtration on a Sephadex G-100 column in 0.05 M sodium phosphate, pH 6.0. Further purification was accomplished either by chromatography on DEAE-cellulose, with elution by a linear gradient from 0.05 M to 0.35 M sodium phosphate, pH 6.0, or by chromatography on Mono Q media, and elution with a linear gradient from 0.05 to 0.35 M ammonium acetate, pH 5.8. In both cases two chromatographically distinct forms of prochymosin were observed.

3. Zymograms

Agar gel electrophoresis was carried out with 1% agar in 0.05 M sodium acetate pH 5.4. Protease containing zones were detected by clotting of casein as described by Foltmann *et al.*[12].

The milk-clotting activity was determined according to Foltmann[5]. The general proteolytic activity was assessed by digestion of acid denatured hemoglobin as described by Nielsen and Foltmann[21].

4. SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotting

SDS-PAGE was performed as described by Laemmli[15] or Schägger and von Jagor[25]. Modifications of the methods for subsequent electroblotting to PVDF membranes and microsequencing were done as described by Ploug *et al.*[22] or Houen[14].

5. Amino acid analysis

Samples were hydrolyzed and analyzed as described by Barkholt and Jensen[1]. Tryptophan was determined by spectroscopy according to Beaven and Holiday[2].

6. Carboxymethylation and carboxamidomethylation

40 nmol of protein were dissolved in 0.5 ml of 6 M guanidine, 0.2 M Tris, 10 mM EDTA, pH 8.4. The solution was flushed with argon and 5mg of DTT was added. After incubation at 37℃ for 2.5 hrs, 13.5mg of iodoacetamide was added and incubation was continued at room temperature in the dark for 1 hr. The reaction was stopped by addition of mercaptoethanol to 1%, and the solution was dialyzed against 50 mM phosphate buffer, pH 7.4, 0.1% SDS (carboxamidomethylated) or 50 mM N-ethylmorpholine acetate, pH 8.4 (carboxymethylated).

7. Proteolytic digestions

All digestions took place overnight at 37℃ using reduced and carboxamidomethylated or carboxymethylated protein. Trypsin and *S. aureus* V8 protease digestions were carried out in 50 mM sodium phsphate, pH 7.4, using enzyme substrate ratios of 1 : 50, 1 : 100 or 1 : 1,000. Digestions with chymotrypsin were carried out in 50 mM N-ethylmorpholine acetate, pH 8.4 using an enzyme substrate ratio of 1 : 100.

All digests were acidified with TFA to pH 1 before chromatography, or were chromatographed immediately after digestion on a 0.2×25cm reversed phase column (5μm, 300 Å, Brownlee Laboratories) or on a 0.4×15cm reversed phase column (Nucleosil, 5μm C-18, Macherey Nagel) using a model 130A HPLC system (Applied Biosystems). The buffers used were : A. 0.1% TFA, B. 80% CH_3CN, 0.09% TFA. Flow : 275μℓ /min. Gradient : 5 min at 100% A, then from 5 to 73% B over 50 min and finally to 100% B over 1 min followed by 100% B for 15 min. The detector was set at 220 nm, and fractions were collected manually.

8. Chemical cleavages

For CNBr cleavage, 5 nmol of protein were dissolved in 100 μℓ of 70% TFA and 100 μℓ of CNBr in 70% TFA (14.2mg /ml) were added. The reaction mixture was flushed with argon, kept in the dark overnight at room temperature, and then taken to dryness *in vacuo*. The fragments were dissolved and separated by gel filtration in 40% acetic acid on a TSK 3000 SW column, by reversed phase HPLC or by SDS-PAGE. In BNPS-Skatole cleavage, 5 nmol of protein were dissolved in 70 μℓ of 80% acetic acid, and 20 μℓ of water and 5 μℓ of BNPS-Skatole in 80% acetic acid (2.3mg /ml) were added. The reaction was allowed to proceed at 37℃ for 22 hrs, and then the reaction mixture was taken to dryness *in vacuo*. Fragments were separated by SDS-PAGE. In limited acid hydrolysis, 5 nmol of protein were dissolved in 200 μℓ of 0.1 M HCl or 200 μℓ of 70% formic acid and kept at 37℃ overnight. The mixture was taken to dryness *in vacuo* and the fragments were separated by SDS-PAGE.

9. Amino acid sequencing

Samples were sequenced on a model 477A protein sequencer (Applied Biosystems, Foster, USA) using chemicals and programs supplied by the manufacturer.

10. Cloning and nucleotide sequencing

Where nothing else is indicated all procedures followed standard protocols as given by Maniatis *et al.*[17] and Sambrook *et al.*[23].

A cDNA library was constructed form the stomach mucosa of a newborn dog using a mRNA purification kit (Pharmacia) for isolation of poly A mRNA and a cDNA synthesis kit (Pharmacia) for cDNA synthesis followed by ligation with Eco RI /Not I adaptors. All pro-

cedures were carried out according to the manufacturers' recommendations. The cDNA was inserted in the pUC 18 vector's Eco RI site and used for transformation of *Escherichia coli* X1-2 blue. Transformation was accomplished by the calcium method as described by Maniatis *et al.*[17]. Plasmid purifications were done using Qiagen columns and the reagents supplied by and procedures described by the manufacturer (Qiagen, Düsseldorf, Germany). The library was screened using a bovine chymosin full length cDNA probe labeled with [α-^{35}S]ATP using a *random primers labelling kit* (Gibco BRL, Life Technologies, Copenhagen, Denmark). Several clones were isolated and the longest clone was subjected to sequencing in both directions using a combination of walking primers and subcloning of the cDNA with use of the M13/pUC sequencing primers (New England Biolabs, Beverly, USA). All sequencing reactions were performed using the Sequenase system (United States Biochemical, Cleveland, OH, USA) and following instructions supplied with the kit. Sequencing gels (6% polyacrylamide, denaturing buffer gradient gels) were prepared as described by Sambrook *et al.*[23]. Oligonucleotides were obtained from Takara Laboratory (Otsu, Japan).

Ⅲ. RESULTS

1. Electrophoretic heterogeneity

Fig. 1 illustrates the electrophoretic heterogeneity of prochymosin and chymosin in extracts of single newborn dog stomachs. In most of the extracts chymosin is present together with prochymosin. The majority of animals show two major bands of both prochymosin and chymosin, but minor amounts of components with mobilities that are lower or higher than those of the main components are also observed in some

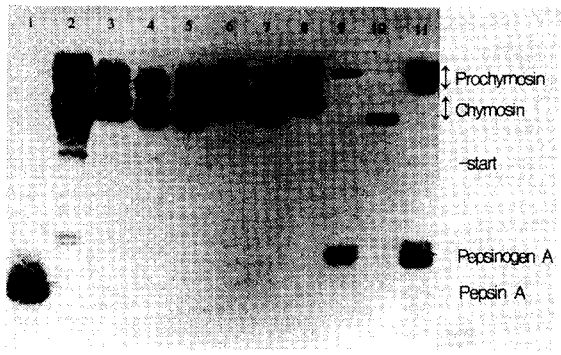

Fig. 1. Agar gel electrophoresis of individual newborn dog stomach extracts as seen in zymograms developed by the clotting of casein. The apparent cationic mobility of prochymosin and chymosin is due to endosmotic flow in the agar gel (anode at the bottom of the figure). According to the recommendations of IUB the isozymes are numbered with the most anionic component as Number 1, and this way the two most predominant components are numbered 2 and 3. Lanes : 1, porcine pepsin A ; 2, mixed extracts used for the chromatographic fractionations : 3-11, extracts of individual dog stomachs of the following ages : 3-8, less than 1 day ; 9 and 10, one week ; and 11, two weeks.

extracts. The differences in the ratios between the contents of zymogens and active enzymes reflect variations in production of gastric acid in the individual animals. This problem was not investigated further. The extract run in Lane 9 contains only zymogens, whereas that in Lane 10 shows active enzymes only. The two samples are included because each shows only single bands of prochymosin and of chymosin, indicating that such individuals were homozygotic with respect to this gene. The presence of minor amounts of pepsinogen in animals aged one and two weeks are consistent with the results of Sangild *et al.*[24].

2. Purification

Canine chymosin was purified from clarified extracts of stomachs from newborn dogs by two consecutive ion exchange chromatographies, which separated several components with milk clotting activity[7]. The chromatographically distinct components with milk clotting activity corresponded to the genetic variants of chymosin also observed in the caseograms of individual stomach extracts. When analyzed by SDS-PAGE all variants had the same relative molecular weight of 35.6 kDa.

3. Enzymatic properties

In spite of several attempts we never obtained a preparation of newborn dog prochymosin that appeared homogenous by SDS-PAGE or amino-terminal sequencing, but immunoelectrophoresis as well as zymograms showed that no other zymogens or enzymes were present. Thus, the preparation of prochymosin was used for activation experiments at pH 2.0 and 4.5, and the development of activity was observed by clotting of reconstituted bovine skim milk. In principle, the activation occurs as for bovine prochymosin[4], but it is noteworthy that at pH 2.0 and 0℃ newborn dog prochymosin developed only about 45% of the maximum milk clotting activity after 2 hrs, whereas that of bovine prochymosin was 100% under the same conditions. With the chromatographically purified canine chymosin qunatitative determinations have been made (Table 1). At the pH for optimal activity (3.2) the general proteolytic activity of canine chymosin was 16% of that of bovine chymosin, whereas its milk clotting activity against bovine milk was 26% of that of bovine chymosin. The maximal general proteolytic activity of canine chymosin is about 4% of the maximal activity of porcine pepsin A. We did not observe any differences in the activities of the individual genetic variants of canine chymosin.

Table 1. Comparison of the enzymatic properties of purified chymosins

Enzyme	Milk clotting activity (% of calf chymosin)	General proteolytic activity (% of porcine pepsin A)
Calf chymosin	100	25
Canine chymosin	26	4
Porcine pepsin A	25	100

4. Amino acid sequence and nucleotide sequence

Amino acid sequencing was cayrried out with the mature enzyme. By direct amino-terminal sequence analysis 22 residues were unequivocally identified (Fig. 2). The amino-terminal sequence has two prolines at position 25 and 26, after which the yields of phenylthiohydantoin amino acids became very low, yielding only a few uncertain residues. Additional sequence information was obtained in two different ways. One approach used fragmentation of reduced and carboxymethylated substrate by enzymatic and chemical methods followed by isolation of the resulting peptides by HPLC. Considerable solubility problems were encountered when canine chymosin was cleaved and the fragments subjected to chromatography. As another approach to obtain sequence information we used specific chemical cleavages in combination with SDS-PAGE, electroblotting to PVDF membranes and sequencing of distinct bands. All in all, sequencing at the amino acid level provided us with 192 residues.

To obtain the full sequence we used a full length cDNA probe for bovine chymosin to

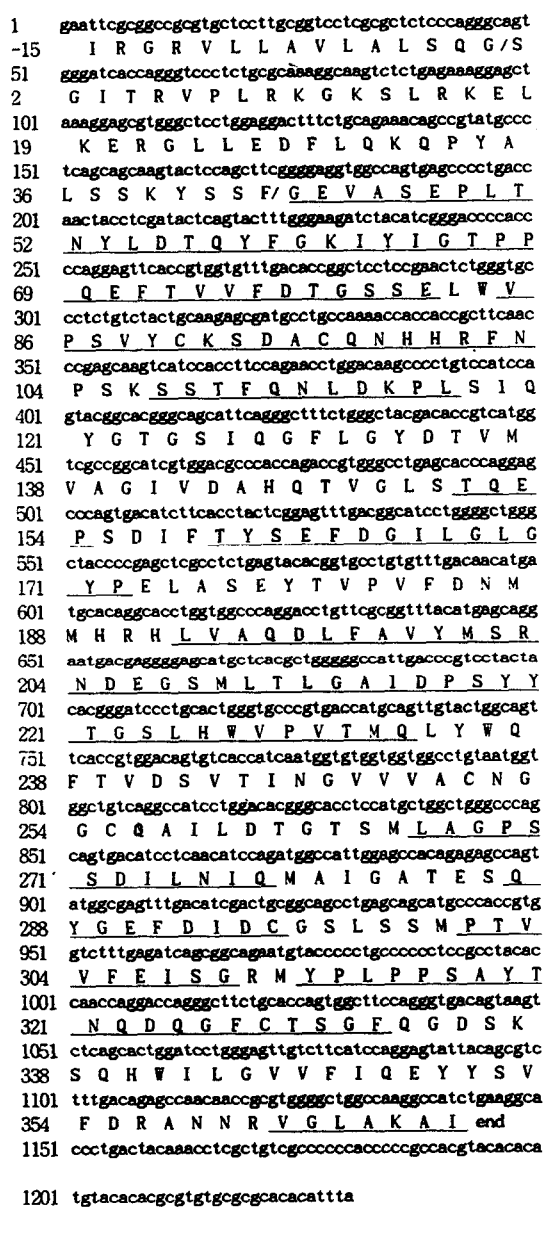

Fig. 2. The cDNA sequence of canine prochymosin and part of its signal peptide. The derived amino acid sequence is also presented. The cleavage points between the signal peptide and the propart is inferred from homology with calf chymosin. The cleavage point between the propart and the active enzyme was established by N-terminal sequencing. Both cleavage points are marked by a /.

screen a cDNA library constructed from a newborn dog stomoch mucosa. The longest clone isolated was sequenced in both directions using a combination of walking primers and subcloning after deletions of the cDNA. This yielded the sequence shown in Fig. 2 together with its derived amino acid sequence. Residues which were confirmed by amino acid sequencing are underlined. The clone covers all of the enzyme, its propart and part of the singal sequence.

5. Amino acid composition

The amino acid composition of chromatographically purified and electrophoretic homogenous preparations of the two genetic variants of canine chymosin was determined. Within an experimental error of 2~3% it was impossible to observe any differences between the two, and both compositions were consistent with the sequence derived composition. Furthermore, no signs of phosphorylation or glycosylation in the form of phosphoserine, phosphothreonine, glucosamine or galactosamine were observed in either short-term (1~2hrs) or in 24hrs hydrolyses.

We also calculated A_{280} (0.1%) for the two genetic variants. A value of 1.46 was obtained for each variant. The calculated molecular weight of canine chymosin is 35,385 Da, which agrees well with the value of 35,600 observed in SDS-PAGE.

Ⅳ. DISCUSSION

Like many other gastric proteases canine

Residues conformed by direct N-terminal sequencing, by sequencing of peptides from reversed phase HPLC or by sequencing of electroblotted peptides separated by SDSPAGE are underlined.

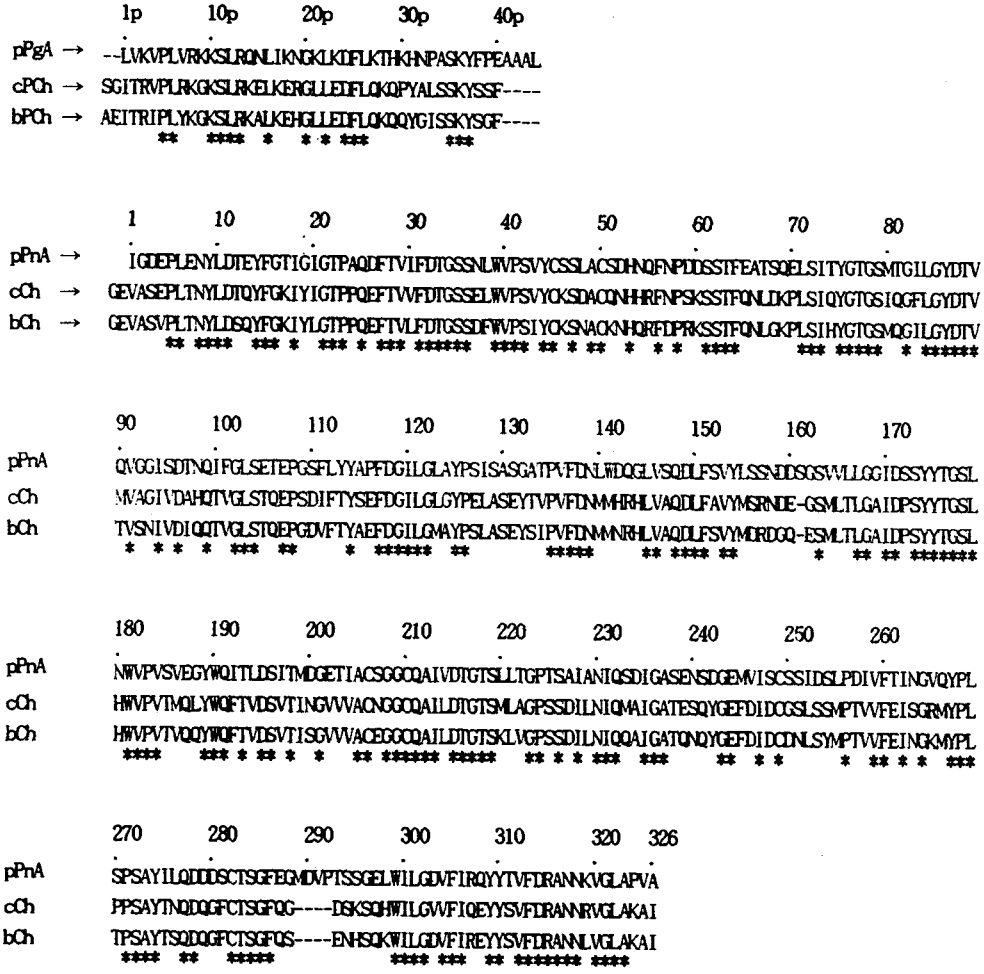

Fig. 3. Alignment of the amino acid sequences of porcine pepsinogen A (pPgA, pPnA), canine prochymosin (cPCh, cCh), and bovine prochymosin (bPCh, bCh). The proparts are presented in the upper section. In order to facilitate comparison the numbering in this figure follows that of porcine pepsinogen. The suffix p indicates the propart, and the numbering starts from the N-terminus of pepsin. An * indicates identical residues.

chymosin occurs as several genetic variants. The apparent homozygocity in some animals suggests that, as for calf chymosin[19], there is only one cistron for canine chymosin with co-dominant expression of the two alleles in heterozygotic animals. Comparison with the electrophoretic mobilities of human pepsinogens[6] indicates that there is a charge difference of one between the two predominant forms, and since the relative electrophoretic mobilities are maintained after activation of the zymogens, these differences must reside in the mature proteins. The substitutions have not been located by amino acid sequencing, and it was not possible to demonstrate differences in the amino acid compositions of the components. In

this connection it should be recalled that it was not possible to demonstrate the difference between calf chymosin A and B by amino acid analyses ; only sequencing showed the substitution of an Asp to a Gly residue[10]. Fig. 3 shows an alignment of the amino acid sequences of newborn dog and calf chymosin[10,13]. For comparison the sequence of porcine pepsinogen A[16,20] is also included. In order to maintain a consistent numbering of residues that are involved in the catalytic mechanism, the numbering in the alignment is that of porcine pepsinogen and pepsin. This gives rise to some problems in the amino-termini and in the connections between proparts and active enzymes. However, we consider this system the best possible. The identity between the two prochymosin sequences is 81% whereas the identity between newborn dog prochymosin and porcine pepsinogen A is 57%.

The location of the disulfide bridges was not experimentally determined in newborn dog chymosin, but from the high degree of similarity with bovine chymosin ; we consider it to be beyond any doubt that the six cysteines are connected in the same way as in all other gastric proteases (40~50, 206~210 and 249~284 using the porcine pepsin numbering).

The size of the propart and the location of the residue which becomes the amino-terminus in the active molecule are the same in the prochymosins. The activation of canine prochymosin was not investigated to the same extent as the activation of porcine pepsinogen and bovine prochymosin. In agar gel electrophoreses of activation mixtures, however, we did observe components with electrophoretic mobilities between those of prochymosin and chymosin, and we also found that the milk clotting activity of canine chymosin develops more slowly than that of bovine chymosin at pH 2. Investigations on the activation of porcine pepsinogen[21] suggested that the negatively charged Glu residue at position 4 was involved in the formation of non-covalent activation intermediates with reduced milk clotting activity. Like porcine pepsinogen, canine prochymosin has a Glu residue at position 4, whereas calf prochymosin has a Val residue at this position. This difference between the two prochymosins may explain the differences observed during activation.

Canine chymosin shows a high specificity towards its natural substrate (kappa casein). The residues that participate in the substrate binding are presumably the same as those identified in other aspartic proteases, e.g. *Endothia parasitica* pepsin[3] and porcine pepsin[26]. Of these residues calf and newborn dog chymosin differ in the following positions (porcine pepsin numbering, subsites in parentheses) : Ser12 Thr(S_4), Leu30 Val(S_1/S_3), His74 Gln(S'_2), Val111 Ile(S_1/S_3), Lys220 Met(S_4). Among these positions, residues 111 and 220 have been the object for protein engineered substitutions in bovine chymosin. The mutation Val111 Phe[27,28] led to local changes in conformation with resulting changes in the kinetic constants for hydrolysis of synthetic peptide substrates. The mutant Lys220 Leu[28] showed a marked shift of the optimum pH to the acidic side for hydrolysis of acid denatured hemoglobin along with an increased general proteolytic activity. In canine chymosin the apolar residue at position 220 does not result in a change of optimum pH relative to calf chymosin. With regard to the low general proteolytic activity of canine chymosin, the substitution of Asp 303 Val relative to calf chymosin may contribute to an explanation of this. Most of the aspartic proteases have an Asp residue at position 303, but renin has an Ala residue. As the Asp 303 is hydrogen bonded to

Thr 216, which is involved in the system of hydrogen bonds at the active site, it has been suggested that Asp 303 contributes to the reactivity of the catalytic Asp residues (Asp 32 and Asp 215), and that the low general proteolytic activity of renin is due to the substitution of Asp with Ala at position 303[18].

V. ABSTRACT

Newborn dog chymosin was extracted from the stomachs of dogs of 2 weeks of age, and was purified by ion exchange chromatography. Half of the sequence was determined by amino acid sequencing and the complete sequence was deduced from a cloned chymosin cDNA. Results showed that the zymogen showed 79% sequence identity with calf prochymosin and 54% identity with porcine pepsinogen A. The size of the propart and location of the residue which becomes the amino-terminus in the active enzyme was the same in the prochymosins. The maximum general proteolytic activity at pH 3.2 of newborn dog chymosin was 3~4% of that of porcine pepsin A at pH 2, whereas the milk clotting activity relative to the general proteolytic activity of newborn dog chymosin was much higher than that of calf chymosin. Agar gel electrophoresis at pH 5.2 of stomach extracts of individual dogs showed the existence of two predominant genetic variants of zymogen and enzyme. The two variants could not be distinguished by amino acid composition or amino-terminal sequencing, and no differences in the enzymatic properties of the genetic variants were observed. It was concluded that of the residues that participate in the substrate binding, calf and newborn dog chymosin differ in the following positions (porcine pepsin numbering, subsites in parentheses) : Ser 12 Thr (S_4), Leu 30 Val (S_1/S_3), His 74 Gln (S'_2), Val 111 Ile (S_1/S_3), Lys 220 Met (S_4). With regard to the low general proteolytic activity of newborn dog chymosin, the substitution Asp303 Val relative to calf chymosin may contribute to an explanation of this.

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