Purification, Characterization, and Amino Acid Sequences of Pepsinogens and Pepsins from the Esophageal Mucosa of Bullfrog (Rana catesbeiana)*

(Received for publication, February 25, 1991)

Etsuko Yakabe‡§, Masao Tanji‡, Masao Ichinose‡, Satoshi Goto‡, Kazumasa Miki‡, Kiyoshi Kurokawa‡, Hisashi Ito‡, Takashi Kageyama¶, and Kenji Takahashi‡**

From the ‡Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113, the ¶Department of Chemistry, Faculty of Science and Technology, Aoyama Gakuin University, Setagaya-ku, Tokyo 157, the †First Department of Internal Medicine, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo 113, and the ||Department of Biochemistry, Primate Research Institute, Kyoto University, Inuyama, Aichi 484, Japan

Two pepsinogens (pepsinogens 1 and 2) were purified from the esophageal mucosa of the bullfrog (Rana catesbeiana), and their molecular weights were determined to be 40,100 and 39,200, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The NH₂-terminal 70-residue sequences of both pepsinogens are the same, including the 36-residue activation segment. Furthermore, a cDNA clone encoding frog pepsinogen was obtained and sequenced, which permitted deduction of the complete amino acid sequence (368 residues) of one of the pepsinogen iso-zymogens. The calculated molecular weight of the protein (40,034) coincided well with the values obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These results are incompatible with the previous report (Shugerman R. P., Hirschowitz, B. I., Bhown, A. S., Schroenloher, R. E., and Spenney, J. G. (1982) J. Biol. Chem. 257, 795–798) that the major pepsinogen isolated from the bullfrog esophageal gland is a unique "mini" pepsinogen with a molecular weight of approximately 32,000–34,000.

The two pepsinogens were immunologically indistinguishable from each other and related to human pepsinogen C. The deduced amino acid sequence was also more homologous with those of pepsinogens C than those of pepsinogens A and prochymosin. These results indicate that the frog pepsinogens belong to the pepsinogen C group. They were both glycoproteins, and therefore, this is the first finding of carbohydrate-containing pepsinogens C. Both pepsinogens were activated to pepsins in the same manner by an apparent one-step mechanism. The resulting pepsins were enzymatically indistinguishable from each other, and their properties resembled those of tuna pepsins.

Pepsinogens are the precursor forms of pepsins, which are typical aspartic proteinases normally present in the gastric mucosa of vertebrates ranging from man to fish. They are

secreted into the gastric lumen and activated autocatalytically to pepsins under acidic conditions, releasing the NH₂-terminal activation segments (1–3). There are three major types of pepsinogens, pepsinogen A, pepsinogen C (or gastricsin), and prochymosin (or neonatal pepsinogen), and they have been purified from various mammalian sources and characterized well. The complete amino acid sequences are known for human (4), monkey (5), porcine (6–9), and rabbit (10) pepsinogens, human (11), monkey (12), and rat (13, 14) pepsinogens C, and bovine prochymosin (15). As for non-mammalian pepsinogens, however, detailed studies on molecular and enzymatic properties have been done only for a few species such as chicken (16) and tuna (17), and the complete amino acid sequences are known only for chicken adult (18) and embryoic (19) pepsinogens. From the viewpoint of structure-function relationships and molecular evolution of pepsinogens, it is of interest to purify pepsinogens of other non-mammalian species and investigate their molecular and enzymatic properties in detail, including their amino acid sequences.

Previously, two pepsinogens were isolated from toad (Caudiverbera caudiverbera) gastric mucosa and activated to pepsins, and a number of properties, i.e. pH optima, action on N-acetyl-l-phenylalanyl-l-diiodotyrosine, and alkaline stability, were briefly investigated (20). Furthermore, one major pepsinogen was isolated from the esophageal glands of the bullfrog (Rana catesbeiana), and its molecular weight and amino acid composition were reported (21). In the latter study, the molecular weight of bullfrog pepsinogen was estimated to be ~31,500–33,700, which is about 25% lower than those of ordinary pepsinogens. Based on these results, bullfrog pepsinogen was concluded to be a unique "mini" pepsinogen, which was assumed to have been formed by deletions in the archetypic pepsinogen late in evolution, thus being of great interest from the standpoint of molecular evolution. Since then, however, no further studies have been reported on bullfrog pepsinogen. On the other hand, physiological studies on pepsinogen secretion have been done mainly using the bullfrog. Therefore, it is important to elucidate in detail the molecular and enzymatic properties of bullfrog pepsinogen.

In the present study we purified two pepsinogens from the esophageal mucosa of the bullfrog (R. catesbeiana) and investigated their molecular properties together with the enzymatic properties of the corresponding pepsins. Furthermore, a cDNA clone of bullfrog pepsinogen was obtained and sequenced, which permitted us to deduce the complete amino acid sequence of one of the pepsinogens. These results showed that bullfrog pepsinogen belongs to the group of pepsinogens.
Frog
PG1
PG2

Human
PGA5

FIG. 1. Time course of activation of pepsinogens 1 and 2 analyzed by SDS-PAGE. Pepsinogens 1 and 2 (each 32 μg) were acidified to pH 2.0 by the addition of a 0.25 volume of 0.1 M HCl and incubated at 14 °C. Aliquots of the reaction mixture (5 μg of protein) were withdrawn at appropriate intervals, mixed with 0.04 M Tris/HCl buffer, pH 8.2, containing 10% 2-mercaptoethanol, 5% SDS, and 37% glycerol, heated at 90 °C for 2 min, and then subjected to electrophoresis. The concentration of acrylamide was 15%. The gel was stained with Coomassie Brilliant Blue R-250. For comparison, activation of human pepsinogen A-5 (PGA5) was also performed under the same conditions. The reaction time (in min) is indicated above each lane. PG, pepsinogen; P, pepsin; I, intermediate form.

C and has almost the same molecular size as ordinary pepsinogens, thus being incompatible with the occurrence of a mini-pepsinogen in the esophageal mucosa.

EXPERIMENTAL PROCEDURES AND RESULTS

Activation Profile and NH2-Terminal Amino Acid Sequences of Pepsinogens—The activation of each pepsinogen was analyzed by SDS-PAGE as shown in Fig. 1. The activation profiles of frog pepsinogens 1 and 2 were essentially the same. Under the conditions used, activation was rapid and complete within 5 min to produce the corresponding pepsins directly. No intermediate protein species was found. On the other hand, the activation of human pepsinogen A-5 (PGA5) (22), used for comparison, proceeded through an intermediate form, and it took 120 min for complete conversion of pepsinogen to pepsin.

To analyze the process more clearly, pepsins from both pepsinogens and the activation peptides from pepsinogen 1 were isolated by Mono Q column chromatography and HPLC (data not shown). Analyses of the NH2-terminal amino acid sequences of the pepsinogens and pepsins and the amino acid sequences of some of the activation peptides (i.e. AP1-1, -2, and -3) established the NH2-terminal 70- and 78-residue sequences of pepsinogen 1 and 2, respectively, as shown in Fig. 2. The results revealed that the NH2-terminal 70-residue sequence of both pepsinogens is identical, and that the entire activation peptides (i.e. propeptides) of pepsinogens 1 and 2 are both composed of 36 amino acid residues. Although some shorter activation peptides including AP1-1, AP1-2, and others were produced by further cleavage of AP1-3 (i.e. the 36-residue intact activation peptide), these cleavages did not appear to take place in the early phase of activation since NH2-terminal analysis of such an activation mixture revealed only the NH2-terminal amino acid residues of the pepsinogens and pepsins (data not shown). These results together with those obtained by SDS-PAGE indicated that activation took place essentially by a one-step mechanism. Although the activation peptides were not isolated and analyzed for pepsinogen 2, the same results would be expected for this isozymogen since both pepsinogens have the same NH2-terminal sequence, including the sequence of the full activation segment, and since both pepsins have essentially the same enzymatic properties as described below.

Amino Acid Sequence of Pepsinogen Deduced from Its cDNA—The complete amino acid sequence of one of the frog pepsinogens was deduced by nucleotide sequencing of its complementary DNA (cDNA). Total RNA was extracted from frog esophageal mucosa by the guanidine-CsCl method (23-25) and cross-hybridization of the mRNA with a synthetic 45-mer oligonucleotide probe corresponding to an active site segment of the human pepsinogen A gene (4) was confirmed by Northern blot analysis (data not shown). Poly(A) RNA was purified from total RNA by oligo(dT)-cellulose column chromatography (26) and reverse-transcribed into cDNA. The cDNA was inserted into the λgt10 arm at the EcoRI site to make a cDNA library of frog esophageal mucosa (27). Thirty-five positive clones were identified among 7,700 plaques of the cDNA library using the 45-mer probe, and the cDNAs in six of these positive clones were isolated to be inserted into pUC18 plasmid DNA at the EcoRI site. The restriction site map was constructed for one of the 35 positive clones, and nucleotide sequencing was performed to give the complete nucleotide sequence shown in Fig. 3. The cDNA thus isolated and analyzed is composed of 1,367 nucleotides and codes for the full length of the pepsinogen molecule including a 16-residue signal sequence, a 36-residue activation segment, and a 332-residue pepsin sequence. The amino acid sequence deduced from the nucleotide sequence was fully consistent with the NH2-terminal sequence of the zymogens determined at the protein level, and the deduced amino acid composition for the pepsinogen agreed well with the values determined for the proteins. The molecular weight of frog pepsinogen calculated from the deduced sequence is 40,034, which is almost equal to the values obtained by SDS-PAGE of frog pepsinogens. When the amino acid sequence of frog pepsinogen was compared with those of some typical pepsinogens, the identities in frog pepsinogen were 51.0% with human pepsinogen A, 64.5% with human pepsinogen C, 47.5% with chicken pepsinogen, and 43.0% with bovine prochymosin. The sequence also showed that the locations of and the sequences around the two active-site aspartic acid residues.
asparagine residue starts from the NH-terminal isoleucine residue corresponding to the nucleotide sequence derived from pepsinogen. The peptidase and the locations of the three disulfide bonds are well conserved and that there is one potential N-glycosylation site at residue 94 of frog pepsinogen.

**DISCUSSION**

The two major pepsinogens obtained from bullfrog esophageal mucosa were very similar isozymes although they were separable by ion exchange chromatography and native PAGE. The NH-terminal 70-residue sequences were identical, and their molecular weights and amino acid compositions were almost indistinguishable. Furthermore, their activation profiles and enzymatic and immunological properties were essentially the same insofar as examined. At present, it is not very clear whether they are the products of two different genes or of a single gene with different posttranslational modifications. Prior treatment of crude mucosal extract with neuraminidase did not change the native PAGE patterns of the two pepsinogens, indicating that they do not contain sialic acid and hence that the carbohydrate moieties have no electric charge. Therefore, the neutral carbohydrate moieties present in low amounts (i.e., 1.3–1.5 mol/mol) could not be the cause for the differences between the two pepsinogens. Furthermore, both pepsinogens were devoid of phosphate. These results imply the presence of two isozymes with one or a few amino acid substitutions that would affect the net electric charge and hence indicate the presence of two corresponding genes.

The amino acid sequence deduced from the nucleotide sequence of one of the cDNA clones of frog pepsinogens was composed of 368 amino acid residues, and the molecular weight of this polypeptide was calculated to be 40,034. This value is close to those obtained by SDS-PAGE and does not agree with those obtained by gel filtration, indicating that the molecular weight was underestimated by gel filtration. This may be due to the glycoprotein nature of the pepsinogens. Previously, Shugerman et al. (21) purified the major pepsinogen from the esophageal mucosa of the same kind of frog (R. catesbeiana) and determined its molecular weight to be approximately 32,000-34,000 by gel filtration, SDS-PAGE, and sedimentation equilibrium. Based on these results, they assumed that bullfrog pepsinogen is a unique mini-pepsinogen with a molecular weight about 25% lower than those of ordinary pepsinogens. In the present study, however, we could not find a pepsinogen of such a low molecular weight. It seems that the molecular weight of the pepsinogen was underestimated in their study not only by gel filtration but also by SDS-PAGE and sedimentation equilibrium for some unknown reason. Taken together, we are led to conclude that there is no mini-pepsinogen in the bullfrog esophageal gland.
although the possibility cannot be completely excluded that the mini-pepsinogen exists in minute quantity.

Amino acid sequence comparisons show that frog pepsinogen resembles pepsinogens C more than pepsinogen A. Frog pepsinogen was also shown to be immunologically related to pepsinogen C rather than pepsinogen A. These results indicate that frog pepsinogen belongs to the pepsinogen C group.

Although it appears to have diverged markedly after separation from mammalian pepsinogens C. To our knowledge, this is the first finding of a carbohydrate-containing pepsinogen C. The amino acid sequence of the NH₂-terminal activation segment of frog pepsinogen is similar to that of tuna pepsinogen 3 (17) as shown in Fig. 5, and the pH profiles of activity and the susceptibilities toward pepstatin of frog pepsins also resembled those of tuna pepsin 3 (17). These results suggest that tuna pepsinogen 3 may also be classified as a pepsinogen C.

The evolutionary relationships of frog pepsinogen to other pepsinogens were investigated by constructing a phylogenetic tree of gastric aspartic proteinases and cathepsin D, using the amino acid sequences of their activation segments (Fig. 5) as shown in Fig. 6. Indeed, the tree shows a rather close relationship between frog pepsinogen and tuna pepsinogen 3. However, in this tree the frog and tuna pepsinogen group appears to have diverged simultaneously from the common ancestral protein of the pepsinogens C group, the pepsinogen A and prochymosin group, and the cathepsin D group. It is difficult to correctly estimate the branching points of these groups in the early period of evolution. Therefore, it is possible that the pepsinogen C group including frog and tuna pepsinogens first diverged from the other groups, soon to be followed by the separation of the mammalian pepsinogen C group and the amphibian and fish pepsinogen C group. To clarify this point further, the construction of a phylogenetic tree based on the complete amino acid sequences of various pepsinogens including those of tuna pepsinogens will be necessary. Such an attempt is now in progress.

Acknowledgments—We thank Eiji Takayama and Satoshi Ishimaru (Department of Biophysics and Biochemistry, Faculty of Science, The University of Tokyo) for their technical advice and helpful discussions.

REFERENCES


25. Chigwine, J. M., Przybylska, A. E., MacDonald, R. J., and Rutter, W. J.
Bullfrog Pepsinogens and Pepsins

22440

(1979) Biochemistry 18, 5294-5299


Analysis of time course of activation of Pepsinogens

Phosphate bound to protein was determined by the method of Fiske and Subharow (1925). Phosphate was released by heating the samples in 3 M HCl for 1 h and determined by the method of Fiske and Subharow (1925). Phosphate was released by heating the samples in 3 M HCl for 1 h and determined by the method of Fiske and Subharow (1925).
Bullfrog Pepsinogens and Pepsins

DNA sequencing

Six positive cDNA clones were inserted into the EcoRI sites of pHX4 and one of these was digested with EcoRI plus PstI and BamHI plus Sall for mapping. The DNA fragments obtained in these digests were transferred into NUC-10 and NUC-25 and the single-stranded DNA was used for sequence analysis (39,47). DNA sequencing was performed by the dideoxy chain-termination method of Sanger (18) with sequencing version 4.1 (19).

Northern blot hybridization

Poly(A)-RNA and total RNA were analyzed essentially according to Sambrook et al. (10). Hybridization was performed at room temperature using a blunt-digoxigenin probe complementary to the active site sequence of human pepsinogen II-5, which was labeled at the 5' terminus with [32P]dATP by T4 polynucleotide kinase.

RESULTS

Purification of pepsinogens

The analysis of the crude extracts of the esophageal and stomach mucosa by native PAGE followed by activity staining (33) revealed the presence of the major and minor pepsinogen components in both mucosa (Fig. 7). The pepsinogen components of the esophageal mucosa were indistinguishable in electrophoretic mobility from those of the stomach mucosa, so they were not separable. The pepsinogen in esophageal mucosa was approximately 8 times higher than that in the stomach mucosa on a weight basis. Accordingly, the following purification of pepsinogen was performed with esophageal mucosa. The PAGE pattern did not change when the crude extract of the esophageal mucosa was treated with neuraminidase prior to PAGE (data not shown).

Pepsinogen in the crude extract of the esophageal mucosa were precipitated by ammonium sulfate between 5-15% saturation, and then purified by successive column chromatography on DEAE-cellulose, Sephadex G-200, and hydroxyapatite (Fig. 8). The pepsinogen components were clearly separated into two major fractions, which were named pepsinogens P1 and 2 in the order of elution from the column as shown in Fig. 8C. Pepsinogen 2 was shown to correspond to the active and the faster-moving components, respectively, found by native PAGE in the crude extract of the esophageal mucosa (Fig. 7).

![Fig. 7. PAGE of the crude extracts of frog esophageal (a) and stomach (b) mucosa. Esophageal mucosa of bullfrog was homogenized with a small volume of 0.1 M sodium phosphate buffer, pH 7.0, by using a Potter homogenizer. The homogenate was centrifuged at 12,000 × g for 20 min. Stomach mucosa was also treated in the same way. Each supernatant obtained from the same weight of mucosa was submitted to PAGE. Electrophoresis was performed according to the method of Laemmli (33) using 10% acrylamide gel and Tris-glycine buffer, pH 8.9. The gel was stained with Coomassie blue with hemoglobin as a substrate as described (34). The figure represents about 70% of the total distance traveled by hemoglobin blue.](image)

![Fig. 8. Fractionation of frog pepsinogens by chromatography on DEAE-cellulose (a), Sephadex G-200 (b), and Sepharose fast flow (c). (a) The sample from the ammonium sulfate fraction step was applied to a column (1.8 × 10 cm) of DEAE-cellulose equilibrated with 0.02 M sodium phosphate buffer, pH 7.0, and eluted with a linear gradient of salt (0-0.5 M) in the same buffer at a flow rate of 0.1 ml/min, fraction size, 20 ml. (b) The sample from the DEAE-cellulose step was applied to a column (3.0 × 30 cm) of Sephadex G-200 equilibrated with 0.02 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5, containing 0.02 M salt, and eluted with the same buffer at a flow rate of 0.1 ml/min, fraction size, 20 ml. (c) The sample from the G-200 column (2.5 × 12 cm) of Sepharose fast flow equilibrated with 0.02 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5, and eluted with a linear gradient of salt (0-0.25 M) in the same buffer at a flow rate of 0.5 ml/min.](image)

### TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>nL / mg</td>
<td>mg / ml</td>
<td>mg / ml</td>
<td>mg / ml</td>
<td>mg / ml</td>
</tr>
<tr>
<td>Crude extract</td>
<td>471</td>
<td>7190</td>
<td>13190</td>
<td>1.4 100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>2196</td>
<td>3849</td>
<td>13200</td>
<td>3.4 101</td>
</tr>
<tr>
<td>5-Sephadex</td>
<td>1566</td>
<td>1515</td>
<td>12590</td>
<td>8.3 95</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>93</td>
<td>604</td>
<td>6900</td>
<td>11.5 53</td>
</tr>
<tr>
<td>Sepharose Pepsinogen 1</td>
<td>97</td>
<td>93</td>
<td>1390</td>
<td>14.8 11</td>
</tr>
<tr>
<td>Pepsinogen 2</td>
<td>183</td>
<td>173</td>
<td>2900</td>
<td>16.8 22</td>
</tr>
</tbody>
</table>

*Potential activity.

extract of the esophageal mucosa (Fig. 7). The results of purification are summarized in Table I. Each of the purified pepsinogens gave a single band upon SDS-PAGE (Fig. 9A), showing that they were electrophoretically homogeneous.

Molecular weights of pepsinogens

The molecular weights of the pepsinogens 1 and 2 were determined by SDS-PAGE to be 48,000 and 39,200, respectively (Fig. 9A). Gel filtration by trifluormethanesulfonyl fluoride (TFA)-treated gelatin columns determined the molecular weights estimated by SDS-PAGE (data not shown). This is consistent with the low content of carbohydrate in both pepsinogens as described later. The molecular weights of the corresponding pepsins 1 and 2 were also determined to be 32,500 and 33,000, respectively (data not shown). These results were roughly similar to those obtained with porcine pepsinogen and peptic under the same conditions (data not shown). On the other hand, both frog pepsinogens 1 and 2 were eluted at a position corresponding to porcine pepsinogen as can be seen from Fig. 9B, and the molecular weights of pepsinogens 1 and 2 were determined to be 24,900 and 25,200, respectively. These values were much smaller than those obtained by SDS-PAGE and also that of 41,200 for porcine pepsinogen determined simultaneously by gel filtration. A similar result was also obtained when both pepsinogens were passed through a TFA-treated gelatin column (data not shown). These discrepancies appeared to indicate that the molecular weights of both frog pepsinogens might be greatly underestimated by gel filtration. Indeed, the values obtained from the complete amino acid sequence of frog pepsinogens and peptic deduced by DNA sequencing as will be described later.

Amino acid compositions and carbohydrate and phosphate contents of pepsinogens

The amino acid compositions of the two frog pepsinogens are shown in Table II, which were calculated using the molecular weights obtained by SDS-PAGE (102). The amino acid compositions of both pepsinogen 1 (111) and 2 (111) and the three groups of higher vertebrate pepsinogen. Frog pepsinogens 1 and 2 had amino acid compositions very similar to each other as compared with the three mammalian pepsinogens. The frog pepsinogens contained somewhat smaller
Bullfrog Pepsinogens and Pepsins

22442

Enzymatic properties of pepsins

Enzymatic properties of pepsins were investigated using pepsins that were produced from the corresponding pepsinogens upon acid treatment and purified by Mono-Q column chromatography (Table III). Two pepsins had essentially the same pH profile, but the pepsini made by using pepsinogen A were around 3.8 with hemoglobin as a substrate (Fig. 10). They had higher relative activity (Fig. 2) and between 3 and 5 as compared with porcine pepsin. Thus their pH profiles of activity were similar to that of pepsin C and pepsin A. Both pepsins cleaved rapidly the synthetic peptide, Leu-Leu-Glu-Pro-Ala-Leu-Lys-Arg-Leu-Lys-Pro-Ala-Leu-Lys-Arg-Leu-Lys-Pro-Ala-Leu-Lys-Arg-Leu-Lys, the standard substrate of canine pepsin, except that the second Arg-Leu-Lys-Pro-Ala-Leu-Lys-Arg-Leu-Lys-Pro-Ala-Leu-Lys-Arg-Leu-Lys-Pro-Ala-Leu-Lys-Arg-Leu-Lys was not cleaved. In the pH 1, 2, and 3, the inhibition profile of the two pepsins by pepstatin were essentially the same (Fig. 11) and their sensitivity to inhibition was significantly lower than that of porcine pepsin. The inhibition profile of these pepsins were similar to that of the pH 3, 4, and 5, and the effects of diamonety-DS-carboxylate metal salt (DAN, in the presence of cupric ions), 1,2-epoxy-3-(3,4-dihydroxyphenyl)borane (1,2-EP-3DHB), and 1,2-epoxy-3-(2,3-dihydroxyphenyl)borane (1,2-EP-2,3DHB) aminopeptidase toward the three pepsins was almost equivalent. These susceptibilities of the three pepsins were similar to those observed with the three papers, especially pepsin B (17).

Nomenclature for amino acids

One of the 35 positive clones was confirmed to contain the full length of the cDNA encoding pepsinogen B and was digested with EcoRI, PstI, and BamHI for subcloning in the M13 phage DNA (8, 47) for further sequence analysis. The restriction map of the cDNA analyzed is shown in Fig. 12 and the complete nucleotide sequence that is obtained is shown in Fig. 3.

**Table III**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total Protein Activity</th>
<th>Specific Protein Activity yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
<td>1.4</td>
<td>23.75</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>1.4</td>
<td>26.95</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.54</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>0.77</td>
<td>23.44</td>
</tr>
</tbody>
</table>

Potential activity.

Fig. 10. pH Dependence of activity of pepsinogen 2C and 2b. About 15 μg of pepsinogen 2C was added to an acid denatured hemoglobin solution of various pH values and was incubated at 37°C for 5 min. The clearance of the substrate was measured by absorbance at 280 nm. The activity was taken as 100% at pH 1.2, pepsinogen 2C (---); 2b, pepsinogen 2C (----); and C, pepsinogen 2C (--). The values of activity were assayed at pH 2.0 for 30 min. The pH values are shown as in Fig. 9.

Fig. 11. Inhibition profiles of pepsinogen 2C and 2b by pepstatin. Pepstatin (about 50 pmol) dissolved in DMSO (0.05 M) was mixed with various amounts of pepstatin dissolved in 333 H2O. After incubation at 37°C for 5 min, 700 μl of 2% Hb solution were added. The pH values were adjusted to 1.2, 1.6, 1.64, and 1.68. The activity was assayed at 37°C for 30 min. The values of activity were assayed at pH 2.0 for 30 min.
Bullfrog Pepsinogens and Pepsins

Fig. 12. Effects on pepsin activity of dimethyl-3-morpholine methyl ether (DMME), 1,2-epoxy-3-azoniabicyclo[2.2.2]octane (EABCO), and phenothiazine methyl ether (PMOEE). The reaction mixture contained 100 μg of crystallized pepsinogen in 0.1 M sodium acetate buffer, pH 4.5, and 10 μl of RM buffer and 50 μl of 3 mM sodium-EDTA buffer, pH 7.4. The reaction was performed at 24°C for 6 h (left) or 12 h (right). Aliquots of each reaction mixture were withdrawn at intervals and used for assay of the remaining activity. The symbols are the same as in Fig. 10.

Fig. 13. Sequence analysis strategy of the frog pepsinogen cDNA clone. The coding region is indicated by an open box. The figure indicates the position of restriction sites. The arrows indicate the direction and extent of sequence determinations. The full-length cDNA (clone 3) is flanked by the restriction sites, and the overlapping DNA fragments obtained by digestion of the clone 3 with PstI and HindIII (PstI) and HindIII and HindIII in N13 clones, respectively. The nucleotide sequence of the cDNA clone 3 (GenBank accession number X90820) is available from the National Center for Biotechnology Information (NCBI) with sequence version 2.9 (cDNA sequencing). Sequencing was replicated at least twice, especially for the middle of the coding region.