Comparison of the Proteolytic Specificities of Bovine and Porcine Pepsin A

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Bovine pepsin A inactivates pancreatic ribonuclease through a limited proteolysis the same way as does porcine pepsin A. The specific activity is lower and more dependent on the ionic strength.

The proteolytic specificity of bovine pepsin A has been investigated with B-chain of oxidized porcine insulin as the substrate. The specificity resembles qualitatively that of porcine pepsin but with quantitative differences.

A large majority of all studies on the enzymatic properties of the pepsins from mammalian gastric juice has been carried out with porcine pepsin A (EC 3.4.23.1, reviewed by Fruton¹). Investigations on substrate specificity have shown that in addition to the amino acid residues adjacent to the peptide bond hydrolysed, there are important interactions with amino acid side chains at secondary binding sites up to four residues from this bond.²

Determination of the primary structure of bovine pepsin A is in progress.³ The results show a high degree of homology between porcine and bovine pepsin A, but also that some 10 to 15 % of the amino acid residues are substituted. The question is now to which extent such substitutions may influence the binding of substrate peptide chains to the enzymes.

Anfinson⁴ observed that porcine pepsin would inactivate ribonuclease through a limited proteolysis during which the C-terminal tetrapeptide Asp-Ala-Ser-Val was cleaved off.*

* Abbreviations of amino acids and derivatives as listed in *J. Biol. Chem.* 247 (1972) 997 with the addition of Cys=cysteic acid.

Berger *et al.*⁵ suggested that this reaction could be used as a general assay for pepsin. We have found that bovine pepsin also inactivates ribonuclease, but that the reaction is more dependent on ionic strength.

The B-chain of oxidized insulin has been widely used for testing the specificities of acidic proteinases (reviewed by Hofmann⁶). Since the degradation of ribonuclease was dependent on ionic strength we have tested the degradation of the B-chain at pH 2 and ionic strengths of 0.01 and 0.2. The results show that porcine and bovine pepsin A attack the same peptide bonds but quantitative differences are observed.

EXPERIMENTAL

Materials

The porcine pepsin was a twice crystallized preparation from Sigma (lot 95B-1270) further purified by ion-exchange chromatography on DEAE-cellulose in 0.1 M sodium acetate (pH 5.1), with a linear gradient 0.2–0.5 M NaCl.

The bovine pepsin was prepared from activated bovine pepsinogen by ion-exchange chromatography on DEAE cellulose⁷ or by affinity chromatography on polylysine-Sepharose.⁸

Bovine pancreatic ribonuclease was from Miles Seravac Ltd. (lot 36–512).

Oxidized insulin B-chain was prepared from swine insulin Novo (lot No. MC-S-169 Ac) by the procedure described by Bang-Jensen *et al.*⁹

Ribonucleic acid (Yeast RNA, Fluka) was freed from interfering low molecular weight components by dialysis against 50 volumes of 0.1 M sodium acetate (pH 5.0) for 20 h and twice against 50 volumes of water for 24 h.

Pyridine (Merck, *p.a.*) was distilled over ninhydrin (2 g/l of pyridine) and over potassium permanganate (1 g/l of pyridine). Other reagents were of analytical grade and used without further purification.

Methods

Peptic digestion of B-chain from oxidized insulin. In all experiments reported in this paper 2.4 μ mol of B-chain were digested with 3 nmol of bovine or porcine pepsin in 0.01 and in 0.20 M ammonium formate (pH 2.0). The reaction took place at 37 °C for 3 h and was stopped by addition of a few drops of 25 % ammonia to a final pH of 9.0–9.5. The digests were lyophilized and submitted to ion-exchange chromatography.

Ion-exchange chromatography. The lyophilized digests were dissolved in 525 μ l of 25 % formic acid and 500 μ l were applied to a cation exchange column by means of a sample loop valve. The ion-exchange resin (Aminex A-5, Bio-Rad Laboratories) was packed in a 0.9 $\times$ 24 cm glass column with a water-jacket. Elution took place at 50 °C with 30 ml of 0.01 M pyridine acetate (pH 1.90), followed by a linear gradient from this buffer to 2.0 M pyridine acetate (pH 4.90), total volume 500 ml. The flow-rate was 30 ml/h and fractions of 3.0 ml were collected. From the effluent 10 % were removed for automatic detection with a Technicon Auto-analyzer system. The peptides were detected by the ninhydrin reaction after alkaline hydrolysis with 10 % of NaOH (100 °C for *ca.* 70 min) and neutralization to pH 5.4 with 5.5 M acetic acid. The ninhydrin colour was developed at 95 °C for 5 min and was measured in a colorimeter fitted with a 570 nm interference filter.

Paper electrophoresis and paper chromatography. Whatman chromatography paper No. 3 MM was used for electrophoresis and chromatography. High voltage paper electrophoresis was carried out in liquid cooled tanks (coolants and buffers as described by Ambler,¹⁰ but with addition of 8 % pyridine to the toluene coolant used for pH 6.5 electrophoresis). As internal marker a mixture of Dns-OH and Dns-Arg was used. The electrophoretic mobilities at pH 6.5 were calculated relative to Dns-OH (–1.0); at pH 2 the electrophoretic mobilities were calculated relative to Dns-Arg (+1.0). Butanol-acetic acid-water-pyridine (15:3:12:10; v/v) was used as solvent for paper chromatography. Peptides were located on the paper sheets by staining with cadmium-ninhydrin reagent.

Characterization of peptides. The N-terminal amino acid determination was performed by the dansylation method described by Hartley¹¹ (5 cm $\times$ 5 cm polyamide sheets were used). Amino acid analysis of peptides were performed with a Durrum D-500 amino acid analyzer after hydrolysis with redistilled 6 M HCl in sealed, evacuated tubes at 110 °C for 20 h.

Peptic digestion of ribonuclease. Pepsin solution (100 μ l) was added to 250 μ l of a solution of ribonuclease (3 mg/ml). Reaction took place for 40 min at 30 °C, and was stopped by mixing 100 μ l of the reaction mixture with 2 ml of 0.1 M phosphate buffer (pH 8.0). The pepsin was denatured by continued incubation at pH 8.0 for 15 min at 30 °C, before the ribonuclease assay was carried out.

To identify the degradation product after digestion with bovine pepsin, 200 nmol of ribonuclease were digested with 1 nmol of bovine pepsin in 0.10 M sodium citrate (pH 2.0) for 40 min at 30 °C. After inactivation of the pepsin at pH 8 the mixture was applied to a column of Sephadex® G-25 fine (0.9 cm $\times$ 60 cm).

Assay of ribonuclease. Solutions of ribonuclease were diluted by mixing 100 μ l with 1.4 ml of 0.1 M sodium acetate (pH 5.0). To this 1 ml of a 0.8 % solution of ribonucleic acid in the same buffer was added. After incubation for 30 min at 30 °C the ribonuclease and high molecular weight RNA was precipitated with 0.5 ml of 0.75 % uranyl acetate in 25 % perchloric acid. The mixture was allowed to stand at room temperature for 20 min. After centrifugation, 100 μ l of the clear supernatant were diluted with 5.0 ml of water and the absorption at 260 nm was measured.

To facilitate the comparison of the results we define one ribonuclease inactivation unit (RIU) as the proteolytic activity that will lower the extinction at 260 nm by 0.1 extinction unit under the present experimental conditions for the ribonuclease test. For this calculation we use only the linear part of the curves describing the inactivation of ribonuclease.

RESULTS

Degradation of ribonuclease. Experiments were performed in NaCl/HCl and citric acid/sodium citrate solutions (pH 2.0) with ionic strengths of 0.02 and 0.10. The degradation of ribonuclease relative to pepsin concentration is shown in Fig. 1. The curves show that the two pepsins have different specific activities towards ribonuclease, the bovine enzyme having only 10 % of the activity of the porcine pepsin in 0.02 M chloride. Furthermore it is clear that an increase in ionic strength from 0.02 to 0.10 has an effect depending on the anion present. In chloride solutions increasing ionic strength results in increasing activity. This effect is more pronounced with the bovine enzyme, which in 0.10 M chloride shows 20 % of activity relative to the porcine pepsin. In contrast to this, the activity of both enzymes decreases 10–15 % with increasing ionic strength in citrate solutions.

Fig. 1. The degradation of ribonuclease with porcine and bovine pepsins. pH 2.0.

Ionic strength	Specific activity of pepsin, RIU/mg In NaCl (A)		In Na-citrate (B)	
	Porcine pepsin	Bovine pepsin	Porcine pepsin	Bovine pepsin
0.02	1260(Δ)	120($\circ$)	1270($\blacktriangle$)	200($\circ$)
0.10	3960($\square$)	820($\times$)	1120($\square$)	165($\bullet$)

Fig. 2. Ion exchange chromatography of peptides from B-chain of oxidized insulin. The digestion was performed with porcine pepsin in 0.20 M ammonium formate for 3 h.

Fig. 3. Yields of peptides and degrees of cleavage of peptide bonds in insulin B-chain after digestion 0.01/0.2 M buffer. Above B-chain sequence: Degrees of cleavage of susceptible bonds in 0.01 M

Table 1. Distribution of peptides in peaks from ion-exchange chromatography on peptic digests of insulin B-chain.

Pool	Relative mobility		Peptide
	at pH 6.5	at pH 2	
I	-0.34		Phe ₁ -Glu ₁₃
I	-0.48		Tyr ₁₆ -Phe ₂₄
I	-0.45		Tyr ₁₆ -Phe ₂₅
I	-0.75		Val ₁₃ -Leu ₁₅
II	-0.75		Val ₁₂ -Leu ₁₅
II	-0.89		Val ₁₃ -Ala ₁₄
II	-1.00		Val ₁₂ -Glu ₁₃
III	-0.27		Phe ₁ -Leu ₁₅
III	0	0.57	Phe ₁ -Leu ₁₁
IV	0	0.76	Ala ₁₄ -Leu ₁₅
V	0	0.54	Ala ₁₄ -Tyr ₁₆
VI	-0.14		His ₅ -Leu ₁₁
VII			No peptide, NH ₃
VIII	+0.34		Phe ₂₅ -Ala ₃₀
IX	+0.43		Tyr ₂₆ -Ala ₃₀
X	+0.47		Thr ₂₇ -Ala ₃₀

The elution profile from the experiment, in which ribonuclease digested by bovine pepsin was submitted to gel-filtration, consisted of two peaks only, one at the exclusion volume showing an amino acid composition approximately corresponding to that of total ribonuclease, and a second just preceding the salt volume, absorbing at 220 nm but not at 280 nm. The latter peak had an amino acid composition of 1.04 Asp, 0.82 Ser, 0.96 Ala and 0.99 Val, proving that the same tetrapeptide is released from ribonuclease by degradation with bovine and porcine pepsin. The yield was 150 nmol corresponding to 75 %.

Digestion of B-chain from oxidized insulin. In all four experiments in this series, the elution profile after ion-exchange chromatography was similar to that of Fig. 2, the peptide content of the pools being that listed in Table 1.

with porcine (I) and bovine (II) pepsins for 3 h. Below B-chain sequence: Yields of peptides in (solid line) and in 0.2 M buffer (dotted line).

Some of the pools contained a mixture of peptides. From aliquots of such pools the individual peptides were purified by paper electrophoresis/paper chromatography. Each peptide was then identified by amino acid composition and *N*-terminus. However, it is a general experience that purification of peptides on paper most often gives rather poor yields; so for quantitative determinations of recovery other aliquots of the same pools were hydrolysed and subjected to amino acid analysis. Knowing the peptide composition of each pool (see Table 1) it was in all cases possible to quantify the peptides from the recovery of amino acids characteristic for any given peptide. To compress the presentation of results only one example of detailed calculation is presented in Table 2. In pool I, the yield of the peptide Phe₁-Glu₁₃ was calculated from the histidine and aspartic acid content. The sum of the yields

of peptides Tyr₁₆-Phe₂₄ and Tyr₁₆-Phe₂₅ was calculated from the amount of arginine. From the residual amount of phenylalanine the separate yields of these two peptides were calculated, knowing that Tyr₁₆-Phe₂₄ was to contain only one phenylalanine, whereas Tyr₁₆-Phe₂₅ contains two. The yield of the peptide Val₁₂-Leu₁₅ was based on the alanine content. In pool II the yield of the peptide Val₁₂-Leu₁₅ was calculated from the leucine content, and in pool III the yield of the peptide Phe₁-Leu₁₅ was calculated from the amount of alanine.

In Fig. 3 the yields of peptides in the four experiments are shown below the sequence of insulin B-chain. A dash indicates, that the peptide was present in yields smaller than 2 % of the amount of B-chain originally present, and the yield has not been calculated. The degrees of cleavage of the susceptible bonds are calculated by adding the yields of contributing

Table 2. Recoveries of peptides from a peptic digest of B-chain from oxidized insulin (same experiment as shown in Fig. 2). The first column of pools I, II and III show the total amino acid composition of the pool in nmol, as determined by amino acid analysis after acid hydrolysis. The amounts of material in pools IV, V and VI were less than 2 % of the starting material, and the recoveries of peptides present in these pools have not been calculated.

Peptide	Pool I			Pool II			Pool III			Pool VIII		Pool IX		Pool X	
	Phe ₁ -Glu ₁₃	Tyr ₁₆ -Phe ₃₅	Tyr ₁₆ -Phe ₃₄	Val ₁₂ -Leu ₁₅	Val ₁₂ -Leu ₁₅	Val ₁₂ -Ala ₁₄	Phe ₁ -Leu ₁₅	Phe ₁ -Leu ₁₁	Phe ₂₅ -Ala ₃₀	Tyr ₂₆ -Ala ₃₀	Tyr ₂₇ -Ala ₃₀				
Cys	1 930	435	1 120	500	—	—	890	150	790	—	—	—	—		
Asp	460	435	—	—	—	—	980	150	790	—	—	—	—		
Thr	60	—	—	—	—	—	60	—	—	1 020	—	55	—		
Ser	540	435	—	—	—	—	1 050	150	790	20	80	10	—		
Glu	3 820	870	1 120	500	1 400	180	1 120	300	790	—	—	—	—		
Pro	—	—	—	—	—	—	—	—	—	235	1 025	70	—		
Gly	3 610	435	2 240	1 000	—	55	900	150	790	15	60	10	—		
Ala	1 380	—	—	—	1 400	180	130	150	—	220	1 020	60	—		
Val	3 700	870	1 120	500	1 400	180	1 050	300	790	—	—	—	—		
Leu	3 940	870	1 120	500	1 400	—	2 180	450	1 580	—	—	—	—		
Tyr	1 420	—	1 120	500	—	—	—	—	—	230	1 015	—	—		
Phe	3 170	435	2 240	500	—	—	960	150	790	230	—	20	—		
His	830	870	—	—	—	—	1 940	300	1 580	—	—	—	—		
Lys	—	—	—	—	—	—	—	—	—	240	1 030	70	—		
Arg	1 640	—	1 120	500	—	—	—	—	—	—	—	—	—		
nmol of peptide	435	1 120	500	1 400	110	180	150	790	230	1 025	—	65	—		
% Recovery	18.5	48.8	21.1	59.3	4.7	7.8	6.3	33.6	9.7	43.4	2.7	—	—		

peptides on each side of the actual bond and using the higher of the two values. These figures are indicated above the sequence. (Additional experiments with digestion for 24 h did not show alteration in the cleavage pattern).

DISCUSSION

Bovine and porcine pepsin A will inactivate ribonuclease through the same limited proteolysis, the activity of the bovine pepsin being 10–20 % of that of the porcine enzyme (depending on the ionic strength). The result is consistent with the investigations of Lang and Kassell,¹² who tested the activity of the two enzymes towards synthetic di- and tripeptides as well as hemoglobin and found that bovine pepsin was a far less effective enzyme than was porcine pepsin.

Previous investigations on the specificity of porcine pepsin with the B-chain of oxidized insulin as substrate have been qualitative although Keil and Keilova¹³ attempted a quantitative evaluation. To compare the specific activities of porcine and bovine pepsins a strictly quantitative treatment was required.

The yields of peptides in the present work show that some 80 % of the starting material has been recovered in the identified peptides and no indication of transpeptidation has been observed. The recovery is close to the expected value with the kind of chromatography used, thus assuring that no significant amount of peptide material has gone undetected due to low colour yields in the ninhydrin reaction or otherwise.

The absence of significant tailing in the ion exchange chromatography is illustrated by the low content of cysteic acid, glycine and phenylalanine in pool II as well as the total absence of phenylalanine in pool IX of Table 2.

The results of this work clearly demonstrate that qualitatively the two pepsins show the same kind of specificity, but quantitatively a few significant differences are observed. The present results largely confirm the quantitative figures of Keil and Keilova concerning porcine pepsin with one exception: The Tyr₁₆-Leu₁₇ bond, while hardly being cleaved in the present experiments, is a medium cleavage point in the experiments of these authors. Consequently the adjacent Leu₁₅-Tyr₁₆ bond exhibits a lower

susceptibility in their experiments than in ours. Sanger and Tuppy¹⁴ and Etherington and Taylor¹⁵ on a qualitative basis determined Tyr₁₆-Leu₁₇ to be a major cleavage point and Leu₁₅-Tyr₁₆ to be a minor if any cleavage point at all. Furthermore these two authors have found cleavage at the bonds Phe₁-Val₂ and Gly₂₃-Phe₂₄, in contrast to the findings of Keil and Keilova and us (even after 24 h digestion). In view of our results showing the profound dependence of the peptic cleavage of a specific peptide bond on ionic strength and the anion present it is not surprising that different experimental conditions as for instance the kind of buffer and buffer concentration used will cause differences in the observed cleavage pattern.

One remarkable finding common to all investigations on the cleavage of the B-chain with porcine pepsin is the differences in susceptibility of the two identical bonds Leu₁₁-Val₁₂ and Leu₁₇-Val₁₈, the former being cleaved rather efficiently while the latter is completely resistant. As the oxidized B-chain is supposed to exist in a random chain conformation, we may rule out structural factors as being responsible for the observed differences and assign them to the effect of secondary interaction.

The investigations of Inouye and Fruton¹⁶ on the activity of porcine pepsin towards peptide substrates of the A-Phe-Phe-OEt type, where A included histidine, led them to conclude that a histidine in neighbour position to the sensitive bond might play a specific role in enhancing the susceptibility of this bond. This may be part of the explanation of the effective cleavage of the Leu₁₁-Val₁₂ bond, which does not contain any of the amino acids normally considered to make a highly sensitive bond such as phenylalanine, tyrosine and tryptophan. The cumulative effect of absence of a histidine and presence of an adjacent cysteic acid (carrying a negative charge at pH 2, which is known to considerably inhibit the pepsin activity) may contribute to an explanation of the total resistance of the Leu₁₇-Val₁₈ bond towards peptic activity.

The comparison with porcine pepsin shows that bovine pepsin follows the same pattern of specificity, but its significantly lower affinity to the Leu₁₁-Val₁₂ bond suggests that the possible effect of histidine on the susceptibility

of this bond is less in bovine pepsin than in porcine pepsin.

As the histidine is thought to interact specifically with a subsite on the enzyme with consequent change in catalytic efficiency as mediated through induced conformational changes, it seems most probable that amino acid substitutions in this subsite are responsible for the altered sensitivity to secondary interaction (for the subsite model, see Ref. 17).

As a consequence of the fast cleavage of the Leu₁₁-Val₁₂ bond by porcine pepsin, which liberates an α -amino group on Val₁₂ this enzyme cleaves the nearby Glu₁₃-Ala₁₄ bond only to a limited extent. By contrast, bovine pepsin clearly shows similar affinity for these two peptide bonds.

The differences in extent of cleavage of the bonds Phe₂₄-Phe₂₅ and Phe₂₅-Tyr₂₆ observed with both enzymes most certainly must be ascribed to some as yet undetermined secondary interaction, as the two types of bonds, when situated in peptide-substrates of the general structure Z-His-X-Y-OEt, are cleaved at comparable rates by the porcine pepsin.¹⁶

It is interesting to observe that the degrees of cleavage of peptide bonds in the B-chain with both enzymes are strongly dependent on the ionic strength. Most susceptible bonds show less cleavage in the high buffer concentration, but some show the opposite effect, e.g. the Phe₂₄-Phe₂₅ bond. Some bonds even show different kinds of ionic strength dependence with the two enzymes as for instance the Leu₁₁-Val₁₂ bond.

The studies on substrate specificity of proteolytic enzymes have shown that in many cases extended binding areas with a number of subsites contribute to the kinetic specificity of the enzyme. A satisfactory explanation of the observed specificities must account for the nature of such subsites and the way they interact with the side chains of the peptide substrate. One way of attacking the problem is to look at homologous enzymes which show substitutions in one or more subsites and to study the specificity consequences of such alterations. The elucidation of the three dimensional structure of porcine pepsin is near completion.¹⁸ Such a model may enable us to localize the amino acid residues responsible for subsite interaction in this enzyme. We may then look for similari-

ties and differences in the known sequences of homologous enzymes of mammal and microbial origin with the aim of correlating possible substitutions in the subsites with altered proteolytic specificity.

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