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THE SPECIFICITY OF A BOVINE FIBROBLAST CATHEPSIN D

I. ACTION ON THE *S*-SULFO DERIVATIVES OF THE INSULIN A AND B CHAINS AND OF PORCINE GLUCAGON

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SUMMARY

The specificity of a cathepsin D-like enzyme from bovine fibroblasts (dental pulp) has been examined using porcine glucagon, the insulin A chain (*S*-sulfo), and the insulin B chain (*S*-sulfo) as substrates.

Peptides obtained from exhaustive digests were separated by chromatography and high-voltage electrophoresis and, after elution and hydrolysis, identified by quantitative amino acid analysis. The character of susceptible peptide bonds and the relative rate of their hydrolysis led to the following conclusions:

1. The residues most favorable for hydrolysis are large and hydrophobic.
2. Fully charged amino acids were never found as carboxyl or amino donors of the hydrolyzed bond.
3. Susceptible bonds were at least two residues removed from the N- or C-terminal end of the substrate peptide.
4. Some bonds possessing the above characteristics were not cleaved suggesting hindrance by "residual conformation".

INTRODUCTION

The discovery of cathepsins D, a family of proton-activated intracellular peptide peptidohydrolases in bovine spleen[1] has been followed by many reports of similar activities from a variety of organ extracts[2–5]. The enzyme to be discussed in this communication was discovered in fibroblasts from bovine dental pulp[6] and subsequently purified to homogeneity[7]. Preliminary studies indicated that the enzyme possessed more stringent specificity requirements and a slightly higher molecular weight[8] than spleen cathepsin D. The preliminary nature of the previous specificity determination as well as the lack of a concise definition of the term "cathepsin D" prohibited the classification of the new enzyme[8].

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METHODS AND MATERIALS

The bovine dental pulp cathepsin was purified as previously described[8]. Insulin A and B chains (*S*-sulfo) were purchased from Schwarz/Mann while glucagon (porcine) was a gift from Dr Bromer (Eli Lilly Research Laboratories). All other reagents used were analytical grade.

The digestion of peptide substrates

Insulin A and insulin B (5 μmoles each) were dissolved in a few μl of 50% formic acid and immediately diluted to 1 ml with 0.1 M acetate buffer at pH 4.0. Highly purified dental pulp cathepsin (70 μg) was added to each peptide and the mixture incubated at 37 °C for 18 h. Glucagon (8 μmoles) was dissolved in a small amount of NaOH and then adjusted to pH 4.0 with acetate buffer. The total volume of the glucagon digest mixture was approximately 3.0 ml to which a total of 150 μg of cathepsin was added. Glucagon required a larger volume of solvents because of its tendency to gel at low pH values.

Separation of peptides and amino acid analysis

Subsequent to the digestion period the peptides were lyophilized, redissolved in a small amount of pyridine acetate buffer at pH 3.7 and applied to an 18 inch × 22 inch sheet of Whatman No. 31 M paper. The first separation was effected by electrophoresis in a Gilson Medical Electronics electrophoretor (4000 V, 200 mA, 1 h) using a pyridine–acetate–water buffer (1:10:289, by vol.). Following careful removal of the Varsol and buffer in a stream of air at 60 °C the second dimension was developed for 5–6 h by descending chromatography in water saturated butanol–acetic acid (4:1, v/v). Peptides were detected by a dilute ninhydrin reagent as described by Subramanian *et al.*[9] except that the paper was sprayed rather than dipped into the reagent. Light blue spots appeared after drying at 60 °C in a forced-air drying oven. The spots were cut from the paper and eluted with several changes of $1 \cdot 10^{-2}$ M HCl. Thereafter chromatograms were sprayed with the regular strength ninhydrin reagent and dried in order to test for peptides which might have escaped detection by the dilute reagent. None was found on any chromatogram. The recovered peptides were subsequently dried in an Evapomix (Buchler) and hydrolyzed in redistilled constant boiling HCl in evacuated Pyrex vials. The proportions of amino acids in the peptides as well as the yields of the various peptides were determined by quantitative analysis on a Technicon autoanalyzer using the single column system. Buffers for the analyzer were prepared with deionized water (Hydro Service and Supplies, Inc, Durham, N.C.) and singly distilled methanol. Without additional precaution the "ammonia" base line elevation in the range of the basic amino acids was completely eliminated by the use of the highly purified water.

Bio-Gel p-2 was preswollen for 24 h at room temperature and degassed under vacuum with rapid stirring. The column (2 cm × 180 cm) was poured at room temperature in 0.05 M NH_4HCO_3. The transmittance of the effluent was monitored at 230 nm using a Beckman DBG spectrophotometer coupled with a 10-inch recorder.

RESULTS

Digestion of the S-sulfo B chain of insulin

Prior to experiments the substrate was tested for impurities by the same peptide mapping procedure ultimately used for the separation of the digestion mixtures. Two extraneous spots (about 5% of the total) were observed as impurity by a ninhydrin spray reagent. The impurities were not removed nor their nature determined as they could be easily recognized in subsequent peptide maps of the digest. One of the cysteine residues in the B chain appeared to be present as cysteic acid rather than the *S*-sulfocysteine. This conclusion was based on the fact that *S*-sulfocysteine is reconverted (approx. 80%) to cysteine during acid hydrolysis[10]. Some of the peptides obtained from the enzymatic digest yielded cysteic acid instead. The peptides obtained following exhaustive digestion of the insulin B chain by the dental pulp protease at pH 4.0 in 0.1 M acetate buffer are shown in Fig. 1. The approximate quantities of

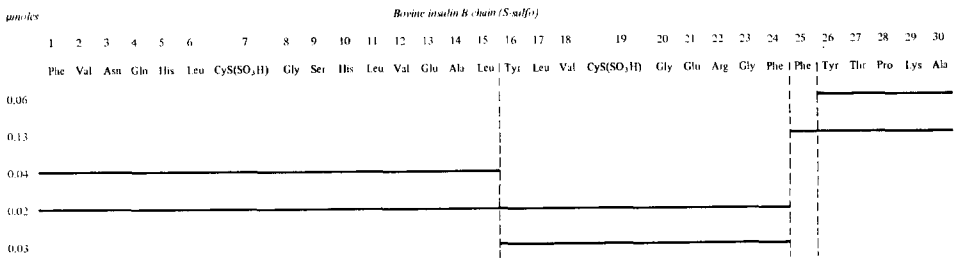

Fig. 1. The peptides isolated from a cathepsin digest of the *S*-sulfo derivative of the bovine insulin B chain. The insulin B chain was dissolved in acetate buffer (pH 4.0) and digested with purified bovine dental pulp cathepsin for 18 h at 40 °C (see Methods and Materials section for details). The types of peptides produced are designated by bars and the total μmoles isolated of each fragment are indicated on the corresponding line to the left. The total micromoles of a certain bond split corresponds to the sum of the peptides with identical (newly produced) carboxyl or amino terminals.

the various peptides produced is an indicator of the relative preference of the proteolytic enzyme for particular types of bonds. Low yields (5–10% of the starting material) can be explained by the partial ninhydrin reaction and adsorption of peptides on the chromatogram. The bovine dental pulp cathepsin shows preference for the three bonds Leu_{15}–Tyr_{16}, Phe_{24}–Phe_{25}, and Phe_{25}–Tyr_{26}. Hydrolysis of additional bonds, known to be hydrolyzed slowly by the uterine cathepsin D[4], could not be demonstrated with the pulp enzyme although the incubation period went over 18 h. (Absence of bacterial contamination was verified by tests on nutrient agar).

Digestion of S-sulfo A chain of insulin by bovine dental pulp cathepsin

The bovine insulin A chain contains a variety of peptide bonds which offer an interesting comparison to similar bonds in the insulin B chain. A Leu–Tyr bond occurs in both peptides, for example, but the neighboring groups are different. The insulin A chain was therefore subjected to hydrolysis by bovine dental pulp cathepsin. The peptide map obtained is depicted in Fig. 2 while Fig. 3 shows the quantitative analysis of various peptides eluted from the chromatogram.

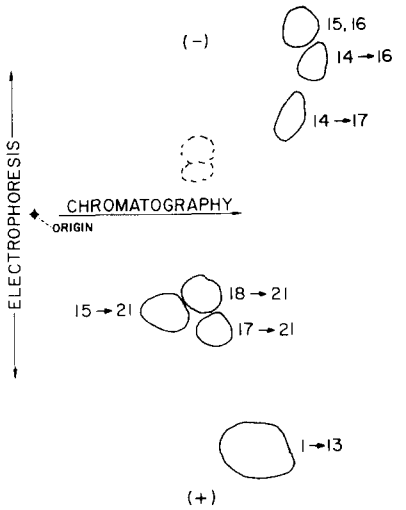

Fig. 2. The peptide map of a cathepsin digest of the *S*-sulfo derivative of insulin A. The digest (5 μmoles) was applied to a Whatman No. 31 M paper (18 inch × 22 inch) and exposed to an electric field (4000 V, 200 mA) for 60 min, followed by descending chromatography in butanol–acetic acid–water. The peptides were detected by a dilute ninhydrin reagent (see text) and traced for photography prior to elution for quantitative analysis. The dotted lines designate weak spots which could not be identified. The numbers next to each spot designate the sequence of the corresponding peptide (compare with the primary sequence, Fig. 3).

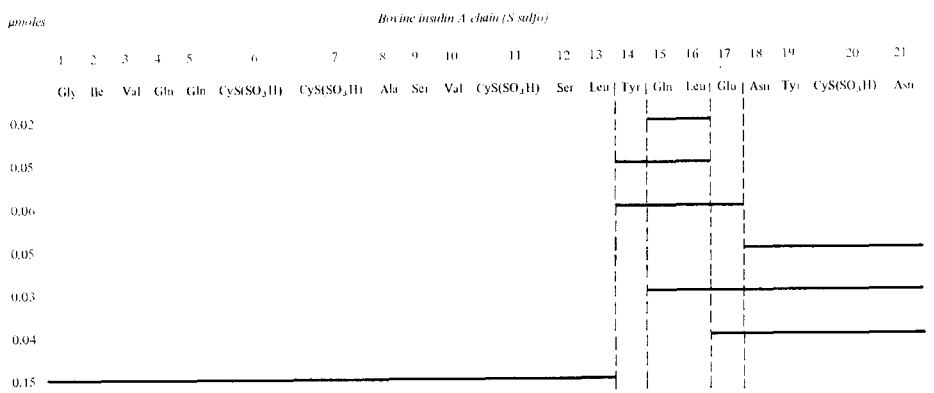

Fig. 3. The peptides isolated from a cathepsin digest of the insulin A (*S*-sulfo) chain. The peptides were eluted from a peptide map (Fig. 2) and subjected to quantitative analysis. See legend of Fig. 1 and text for details.

The hydrolysis of porcine glucagon by the dental pulp cathepsin

Glucagon does not contain cysteine but a single residue of tryptophan. In addition the "sequence isomer" of Leu–Tyr (Tyr–Leu) is formed in an internal position of the peptides. It was therefore decided to examine the action of dental pulp cathepsin on this peptide. Following solubilization of glucagon the enzyme was added immediately and digestion permitted to proceed at 40 °C. Even under these conditions gelling of glucagon could not be totally prevented. The separation and purification of the resulting peptides by high voltage electrophoresis and chromatography alone were

less satisfactory on account of strong peptide–peptide interactions. Prior separation on a Bio-Gel p-2 column (180 cm × 2 cm) in 0.1 M NH_4HCO_3 improved the purification (Fig. 4). The peptides isolated from the digest are depicted in Fig. 5. It is difficult to ascertain the effect of aggregation on the degree of hydrolysis by the fibroblast cathepsin and on the overall yield of peptides. The proportion of the various peptides obtained should still be representative of the relative affinity of the enzyme for certain peptide bonds.

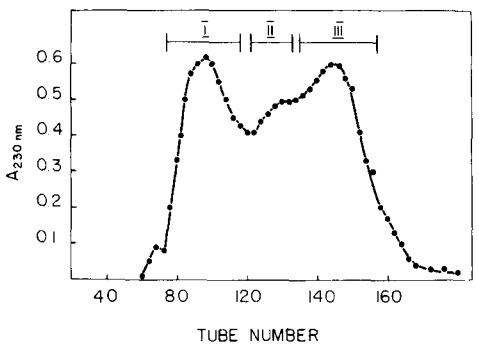

Fig. 4. Elution pattern of a cathepsin digest of glucagon separated on a Bio-Gel p-2 column. The digest was prepared as described for the insulin chains and applied to a 180 cm × 2 cm column and eluted with 0.05 M NH_4HCO_3. Fractions were collected as shown and further purified by high voltage electrophoresis.

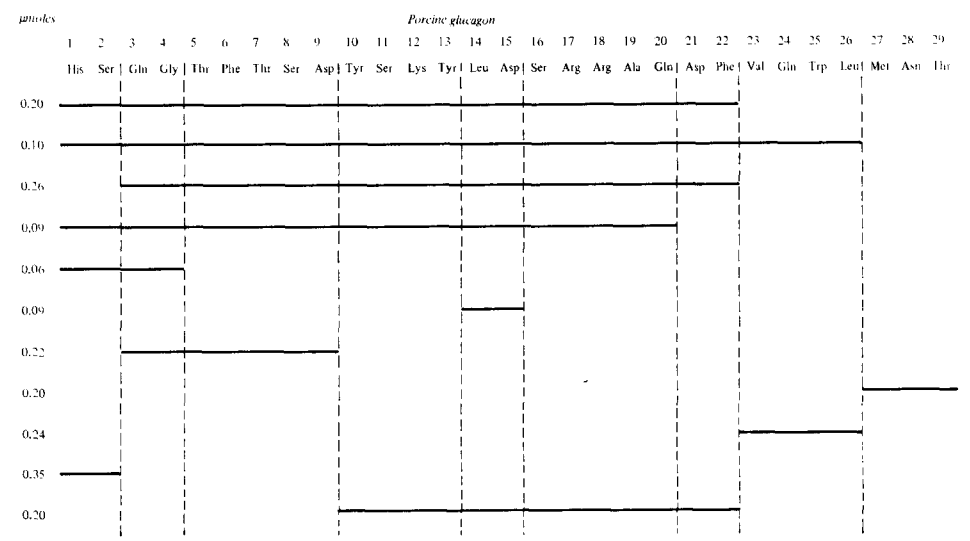

Fig. 5. The peptides obtained from a cathepsin digest of glucagon. Conditions were as described for the insulin chains except that a Bio-Gel p-2 column (Fig. 4) was employed for a preliminary group separation.

DISCUSSION

The specificity of most known proteases is directed toward the amino acid residue contributing either the carboxyl- or the amino group to the hydrolyzable bond. The most specific and perhaps best known enzymes of this group are trypsin and chymotrypsin. While trypsin does not tolerate any substitution for the basic amino acids at the site of action, chymotrypsin will on occasion accept leucine instead of phenylalanine, tyrosine or tryptophan.

Cathepsins D, the lysosomal acid-activated proteases, have often been compared to pepsin with respect to their specificity. However, if a substrate such as oxidized ribonuclease is subjected to extensive hydrolysis by the purified acid cathepsin isolated from dental pulp, the number of fragments obtained is relatively small compared to a trypsin digest of the same substrate[7]. The hydrolysis of insulin B by bovine dental pulp cathepsin is indicative of great affinity of the enzyme for aromatic residues. The bond between Phe_{24}–Phe_{25} is most often attacked followed by the bond Phe_{25}–Tyr_{26} and the bond Leu_{15}–Tyr_{16}. Clearly, the amino donor for the susceptible bonds is an aromatic amino acid in every case while leucine seems to be an acceptable substitute for aromatic acids as the carboxyl donor in some cases.

Conversely, peptides obtained from the digest of the insulin A chain by dental pulp cathepsin show leucine as an excellent carboxyl donor (Leu_{13}–Tyr_{14}). In addition Glu_{17}–Asn_{18} and Leu_{16}–Glu_{17} are good substrates although neither of the components is aromatic. While these residues are largely un-ionized at the pH of the assay (4.0) they cannot be considered hydrophobic in character. The Tyr_{14}–Gln_{15} bond is a site of minor activity for the dental pulp cathepsin. The Leu_{13}–Tyr_{14} bond appears favored considering the total micromoles of the corresponding peptide recovered. Once the bond between residue 13 and 14 has been hydrolyzed, Tyr_{14} becomes the N-terminal amino acid and the bond between Tyr_{14} and Gln_{15} becomes very unfavorable through its position in the remaining peptide chain.

Glucagon is hydrolyzed preferentially at Phe_{22}–Val_{23}. It is plausible that the valine bond in glucagon is hydrolyzed while no enzymatic activity toward the same bond in the N-terminal position of the insulin B chain is observed. Surprising is that the affinity of the uterine cathepsin D is sufficiently great to permit any action at all on the terminal Phe–Val bond of the insulin B chain[4]. The hydrolysis of Ser_2–Gln_3 by the dental pulp enzyme is inconsistent with previous observations, but too extensive to be an artifact. At this time no explanation for the limited hydrolysis of the Gly_4–Thr_5 bond can be given since the small yield of the corresponding peptide makes this result uncertain.

It must be concluded that the structural requirements for a peptide bond to be hydrolyzed by bovine dental pulp cathepsin cannot be specified solely by the features of the carboxyl- or amino donor of the susceptible bond. Bulky groups, hydrophobicity, and the internal position of the sensitive bond are with one (uncertain) exception the only generally recognizable trend. Potential bonds not hydrolyzed by the enzyme have to be considered. Thus in insulin A Ile_2–Val_3, Val_3–Gln_5, in insulin B Leu_{11}–Val_{12}, Leu_{17}–Val_{18}, and to a lesser extent Asn_3–Gln_4, in glucagon Phe_6–Thr_7, qualify under the above criteria, yet are not hydrolyzed. In order to examine the possible influence of neighboring groups susceptible and non-susceptible bonds, including the adjacent residues, are compared in Table I. Evaluating this table it has

TABLE I

AMINO ACID RESIDUES IN THE VICINITY OF THE BONDS HYDROLYZED BY DENTAL PULP CATHEPSIN

Relative amounts	*−3*	*−2*	*−1* ↓ *1*	*2*	*3*
			Glucagon		
1.00	– Gln –	Asp –	Phe –	Val – Gln –	Trp –
0.73	H	– His –	Ser –	Gln – Gly –	Thr –
0.52	– Gln –	Trp –	Leu –	Met – Asn –	Thr – OH
0.30	– Thr –	Ser –	Asp –	Tyr – Ser –	Lys –
0.10	– Arg –	Ala –	Gln –	Asp – Phe –	Val –
0.10	– Ser –	Lys –	Tyr –	Leu – Asp –	Ser –
0.10	– Tyr –	Leu –	Asp –	Ser – Arg –	Arg –
			Insulin B chain ▼		
1.00	– Arg –	Gly –	Phe –	Phe – Tyr –	Thr –
0.46	– Gly –	Phe –	Phe –	Tyr – Thr –	Pro –
0.31	– Glu –	Ala –	Leu –	Tyr – Leu –	Val –
			Insulin A chain ▼		
1.00	– CyS(SO_3H) –	Ser –	Leu –	Tyr – Gln –	Leu –
0.64	– Tyr –	Gln –	Leu –	Glu – Asn –	Tyr –
0.55	– Gln –	Leu –	Glu –	Asn – Tyr –	CyS(SO_3H)
0.27	– Ser –	Leu –	Tyr –	Gln – Leu –	Gln –
			Similar bonds not hydrolyzed		
	– Gly –	Thr –	Phe –	Thr – Ser –	Asp –
	– Ser –	His –	Leu –	Val – Glu –	Ala
	– Leu –	Tyr –	Leu –	Val – CyS(SO_3H) –	Gly –
	H	– Gly –	Ile –	Val – Glu –	Gln –
	H – Gly –	Ile –	Val –	Glu – Gln –	CyS(SO_3H) –

to be considered that the γ- and β-carboxyl groups are partially protonated and that only the protonated form is susceptible to hydrolysis. Thus nine of fourteen residues in the −1 position (Table I) are large and hydrophobic. Of the remaining residues three are dicarboxylic acids, and one is an amide. With the exception of one serine residue, the two most active bonds always have a hydrophobic acid as carboxyl donor. In the 1 position (amino donor) only seven of fourteen residues are strictly hydrophobic, two are dicarboxylic acids, three are the corresponding amides, and the remaining two are serine and methionine. Neither glycine, alanine, cysteine (*S*-sulfo) nor a basic amino acid have been observed at the −1 or 1 position. Only in two instances do arginine or lysine occur in the −2 or 2 position and in both cases the peptide bond is poorly hydrolyzed. The N-terminal histidine in the second peptide of glucagon is a partial exception. Arginine in Position −3 and lysine or cysteine (*S*-sulfo) in Position 3 seem to have little influence.

These considerations are somewhat negated by the lack of activity toward peptides listed in the last category in Table I. Only peptide 3, due to cysteine (*S*-sulfo) in Position 2 can be excluded on the basis of the above arguments.

As shown previously[11] the consideration of auxiliary binding sites interacting with residues removed from the sensitive bond is reasonable. It appears however that

yet another parameter must be considered. It is generally accepted that the denatured protein is not a random coil but has a residual structure which depends on the amino acid composition and the solvent, *i.e.*, ionic strength, pH, and temperature. Our studies here raise the question whether the action of acid-activated intracellular proteinases (perhaps due to their larger structures) is restricted by certain residual conformations which either expose or protect a bond possessing the prerequisit residues as discussed above.

ACKNOWLEDGMENTS

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