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Proteolytic activity of Bothrops protease A on the B chain of oxidized insulin

Bothrops protease A, a heat-resistant, acid-stable proteolytic enzyme, isolated from *Bothrops jararaca* venom, which hydrolyzes gelatin, protamine but not casein[1], exhibits a more selective activity than trypsin on synthetic basic amino acid substrates. It shows hydrolytic activity towards substrates involving the carboxyl group of arginine, synthetic substrates such as benzoylarginine amide and *p*-toluenesulfonylarginine methyl ester, and is not active on lysine ethyl ester[2]. The experiments reported in this paper were done in order to characterize further the proteolytic activity of Bothrops protease A by identifying the peptides resulting from action of the enzyme on the B chain obtained from oxidized insulin. Corresponding experiments with oxidized insulin were also performed.

Hydrolysis of the B chain of oxidized insulin[3,4] was carried out at pH 8.0 and at 37° (enzyme to substrate ratio, w/w, 1:50). High-voltage paper electrophoresis in pyridine–acetic acid–water buffer (pH 3.9) separated the hydrolysate into four fractions, denoted 1, 2, 3 and 4 (Fig. 1). Three of them moved towards the cathode and

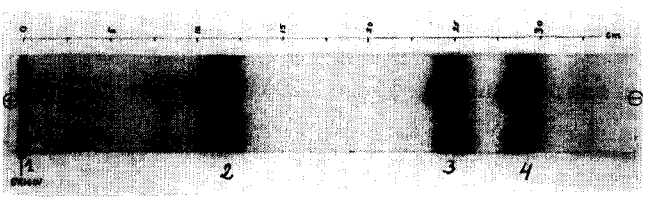

Fig. 1. Fractionation of peptides obtained from Bothrops protease A digest of the B chain of oxidized insulin by paper electrophoresis in pyridine–acetic acid–water buffer of pH 3.9 and 25 V/cm for 4 h. The direction of migration is from left to right towards the cathode.

one (fraction 1) remained at the starting line. When oxidized insulin incubated with the enzyme was submitted to high-voltage electrophoresis, it was observed that the main material remained at the starting line, one peptide moved towards the anode (as also was observed in the electrophoresis control for the oxidized insulin), and three peptides moved towards the cathode and were identified as peptides 2, 3 and 4. This observation demonstrated that Bothrops protease A has no activity on the A chain of insulin.

The purity of these peptides was analyzed. Peptide 1 was shown to be homogeneous, although it always gave an elongated spot when analyzed by paper chromatography with several solvent mixtures; it gave only one peak during chromatography on Sephadex G-25. By paper chromatography in 1-butanol–acetic acid–water (4:1:5, by vol., upper phase), fraction 2 was shown to contain a major component, and also five minor ninhydrin-reacting components. Unfortunately, however, the latter could not be isolated in amounts which permitted further analyses. Peptides 3 and 4 proved to be single components by electrophoresis and chromatography.

The peptides were eluted and half of the material was hydrolyzed with 5.7 M HCl in sealed ampoules for 16 h at 110°, and the amino acids were identified by two-

TABLE I

AMINO ACID COMPOSITION OF PEPTIDES OBTAINED BY THE ACTION OF BOTHROPS PROTEASE A ON B CHAIN OF OXIDIZED INSULIN

Peptides	*Amino acids*	*DNP-amino acid (ether-soluble)*
1	Those found in oxidized B chain of insulin, except Pro, Lys and Thr	DNP-Phe
2	Gly, Phe	DNP-Gly
3	Lys, Thr, Ala, Tyr, Tyr X, Pro	diDNP-Tyr
4	Lys, Thr, Ala, Pro	DNP-Thr

dimensional paper chromatography in 1-butanol–acetic acid–water followed by phenol–sodium borate buffer[5] (pH 9.5). The other half of the eluted peptides was dinitrophenylated according to SANGER AND THOMPSON[3], and analyzed by two-dimensional chromatography on thin-layer plates of silica gel G (BRENNER, NIEDERWIESER AND PATAKI)[6]. The results of this analysis, and the structure of the peptide fractions, are listed in Table I. Peptide 1 was shown to contain all the amino acids of the B chain with the exception of proline, lysine and threonine. Since, on dinitrophenylation, only DNP-phenylalanine was obtained from that fraction (a finding that indicates that it contained only a single peptide), and as the peptide contained arginine, evidence is given of cleavage somewhere between amino acids 22 (arginine) and 26 (tyrosine). However, it can be concluded that peptide 1 resulted from hydrolysis between amino acids 22 (arginine) and 23 (glycine) considering that the peptide 2 showed DNP-glycine as N-terminal and contained only two amino acids, phenylalanine and glycine. On dinitrophenylation, tyrosine proved to be the N-terminal amino acid of peptide 3, which on hydrolysis turned out to be a pentapeptide made up of tyrosine (as well as a small amount of tyrosine X, the formation of which could not entirely be prevented), threonine, proline, lysine and alanine. Consequently, peptide 3 must have been formed by hydrolysis between amino acids 25 (phenylalanine) and 26 (tyrosine) of the B chain of oxidized insulin. The fastest electrophoretically moving peptide (peptide 4) contained the four last amino acids of the B chain of insulin and threonine was recognized as the N-terminal amino acid. It can be concluded from these data that the sites of action of Bothrops protease A on oxidized B chain are those indicated in Fig. 2.

The fact that there was no decrease in peptide 3 with corresponding appearance of free tyrosine during longer periods of incubation, indicated that peptide 4 was not liberated as a subsequent product of hydrolysis of peptide 3 by the enzyme. In fact,

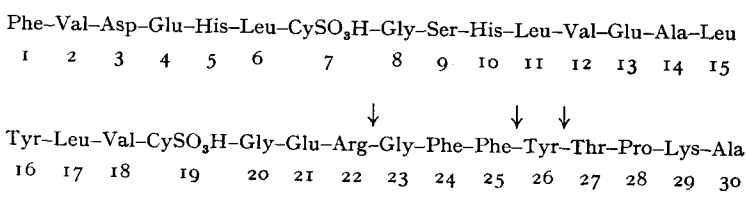

Fig. 2. Summary of the specificity of Bothrops protease A on oxidized B chain of insulin. The arrows indicate the sites of attack by the enzyme.

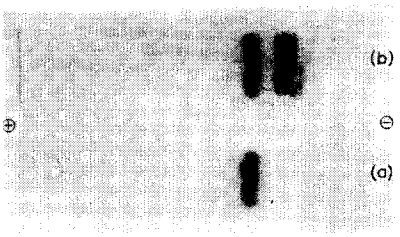

Fig. 3. Paper electrophoresis of Bothrops protease A digest of peptide 3 (Tyr–Thr–Pro–Lys–Ala) in pyridine–acetic acid–water buffer (pH 3.9, and 25 V/cm for 4 h). a, peptide 2 digested with Bothrops protease A; b, the same as (a), but peptide 4 (Thr–Pro–Lys–Ala) was added before electrophoresis.

when peptide 3 (Tyr–Thr–Pro–Lys–Ala) was used as substrate instead of the whole B chain of oxidized insulin, no hydrolytic action was observed on bond 26–27, which involves the carboxyl group of tyrosine. It may be seen (Fig. 3a) that Bothrops protease A was unable to liberate from peptide 3 the tetrapeptide Thr–Pro–Lys–Ala (peptide 4); had the latter been liberated, it would also have been detected, as shown in Fig. 3b, when peptide 4 was added to the incubation mixture before electrophoresis. This negative finding was corroborated by the low hydrolytic activity of Bothrops protease A as compared with chymotrypsin on a synthetic tyrosine ester such as acetyltyrosine ethyl ester. Therefore the action towards the carboxyl group of tyrosine in a peptide bond must be related to a very special peptide configuration[7–9], as only one of the two tyrosine bonds in B chain of oxidized insulin, Tyr–Thr (26–27), was split by Bothrops protease A. This selective action is also shown to be related to the bonds involving basic amino acids: the Lys–Ala (29–30) bond was not hydrolyzed; only Arg–Gly (22–23) bond was split by the enzyme. These results confirm the previous observations on synthetic arginine and lysine derivatives[2].

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