LIMITED ACTION OF TRYPSIN ON PORCINE PANCREATIC AMYLASE : CHARACTERIZATION OF THE FRAGMENTS

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1. Introduction

Porcine pancreatic α-amylase (EC 3.2.1.1:1,4-α-glucan 4-glucanohydrolase) consists of a single polypeptide chain of about 475 residues, with 2 SH groups and 4 disulfide bridges [1,2]. The nine peptides isolated after cleavage of the molecule by cyanogen bromide have been ordered by the use of an isotopic technique [3]. Porcine amylase contains calcium [4,5]. The metal is required for activity and also prevents degradation by trypsin [6]. Two forms of amylase (I and II) with different isoelectric points have been identified [7,8]. In the present work the fragments resulting from trypsin digestion of amylases I and II respectively in the presence of a calcium complexing agent have been identified, purified and characterized.

2. Materials and methods

Amylases I and II were purified from porcine-tissue homogenate [9]. Amylase was assayed by reductometry [10]. The digestion mixture (0.08 mM amylase, 8 mM EDTA, 0.8 μM trypsin, 5 mM sodium phosphate buffer pH 7 and 6 mM NaCl) was incubated for 1 hr at 25°C. Trypsin action was stopped by adding 1 mM di-isopropylphosphorofluoridate (DFP). Peptide hydrolysis (0.05–0.1 μmol) was carried out in evacuated sealed tubes with 1 ml of triple distilled 5.7 M HCl for 24 hr at 115°C. Amino acid analyses were performed by the method of Spackman et al. [11]. Half cystine was determined after performic acid oxidation [12] and after reduction and carboxymethylation of the peptides by iodoacetic acid [13]. SH groups were titrated according to Ellman [14]. The molecular weight of the peptides was calculated from the amino acid composition [15]. N-Terminal amino acids were determined by dansylation [16,17]. C-Terminal amino acids were determined after incubation with carboxypeptidases A and B [18]. Discontinuous gel electrophoresis were carried out according to Ornstein and Davis [19]. Sodium dodecyl sulphate (SDS) gel electrophoresis was performed according to the technique of Shapiro et al. [20]. For determination of the molecular weight of peptides, marker proteins (bovine serum albumin, horse-liver alcohol dehydrogenase, ovalbumin, bovine ribonuclease, horse-heart cytochrome *c*) were used to calibrate the gel. TPCK trypsin was purchased from Worthington. DFP-treated, carboxypeptidases A and B were from Boehringer.

3. Results and discussion

3.1. *Identification of the products at various times of digestion*

Amylase was treated with trypsin (see Materials and methods). Parts of the incubation mixture were taken at various times for amylolytic assay and for SDS gel electrophoresis after reduction and carboxymethylation of the samples. As seen (fig.1) more than half of amylase (top band) was converted, within 15 min, into 2 smaller components, namely peptides A and B (lower bands). At 30 min most of the amylase was degraded into the small components. No further change was observed in the gel electrophoresis pattern.

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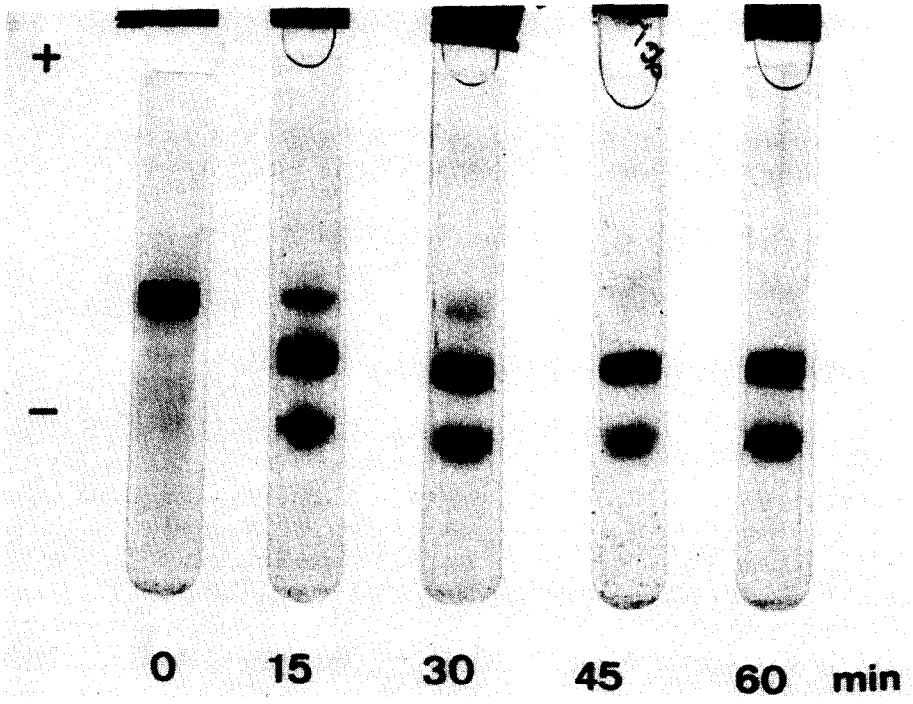

Fig.1. SDS gel electrophoresis patterns of the digestion products obtained at various times of incubation of amylase with trypsin, after reduction and carboxymethylation. The digestion times are as indicated. The composition of the gel was : 5% acrylamide, 0.2% *N-N'*-methylene bisacrylamide, 0.1% SDS. The electrophoretic separation was carried out at 4 mA per tube for 4.5 hr.

The loss of amylolytic activity was parallel to the splitting of the amylase chain [21]. At 60 min the digested enzyme is devoid of any activity. It should be noted that only 2 large peptides, A and B, are produced and are not further degraded. The mol. wts of both peptides, analyzed after reduction and carboxymethylation (A = 35 000 ± 1000 and B = 12 500 ± 1 000) were determined by gel electrophoresis using appropriate protein markers. However the sum of the mol. wts of the peptides (A + B = 47 500 ± 2 000) does not account for the whole amylase molecule (53 000). Small fragments are not detectable by this technique. The digestion products were next analyzed in a more quantitative way.

3.2. *Isolation of the digestion products*

The whole incubation mixture was then filtered on a Biogel P-100 column. As shown (fig.2), 2 peptide fractions were obtained namely P_1 and P_2. P_1 elutes at 1.3 V_o and P_2 at 2.1 V_o. P_1 appears homogeneous not only be gel filtration but also by ultracentrifugation (not shown) and amino acid composition. P_2 is heterogeneous as found by subsequent filtration on Biogel P-10 (not shown).

The amino acid composition of P_1 was determined (table 1) and its minimum molecular weight was calculated [15]. The result (50 100) agrees with the value obtained by sedimentation equilibrium (49 000). P_1 thus appears as a core enzyme. End group analysis by dansylation indicated only one N-terminal residue (Asx), while 2 C-terminal residues (leucine and lysine) were identified by the use of carboxypeptidases A and B. P_1 might thus consist of 2 peptide chains linked by one or several disulfide bridges. This proposal was confirmed by SDS gel electrophoresis of the reduced and carboxymethylated peptide (RCM-P_1) (fig.3).

$$P_1 = A + B$$

Peptides A and B were purified by chromatography of RCM-P_1 on Sephadex G-100 equilibrated with 4 M urea. As shown (fig.3) peptide A elutes with the

Table 1
Amino acid composition of the core enzyme (P_1) and of its 2 peptide chains (A and B)

Amino acid	Number of residues			
	P_1 (His = 9)	A (His = 7)	B (His = 2)	A + B
Asx	59	46–47	14	61–62
Thr	20	13	7	20
Ser	27	20	8	28
Glx	34	23	11–12	34–35
Pro	17	10	8	18
Gly	47	33	13	46
Ala	28	21	7	28
Val	34–35	24	10	34
Cys	10	7	3	10
Met	8	7	1	8
Ile	21	16	5	21
Leu	21	20	4	24
Tyr	16–17	11	5–6	16–17
Phe	21	18	4	22
Lys	18	15	3	18
His	9	7	2	9
Arg	27	17–18	9	26–27
Trp	n.d.[a]	n.d.	n.d.	n.d.
Molecular weight:				
Amino acid composition [15]	50 100	34 360	12 600	
SDS gel electrophoresis	–	35 000 ± 1000	12 500 ± 1000	
Sedimentation equilibrium	49 000	–	–	

[a] n.d. = Not determined.

void volume while B elutes at 1.68 V_0. The amino acid compositions of A and B were determined (table 1). The calculated mol. wts of A and B are in good agreement with the values obtained by SDS gel electrophoresis. Peptide B contains only 1 methionine and 2 histidine residues. Determination of end groups was also carried out on the purified peptides. By dansylation Asx was found at the N-terminal position of A but no detectable N-terminal residue was found in B. The latter N-terminal may then be blocked. By digestion with carboxypeptidases A and B, leucine was found at the C-terminal of A and lysine at the C-terminal of B. Identical results were obtained using amylases I and II.

3.3. *Properties of the core enzyme (P_1)*

The properties of P_1, which has a mol. wt close to amylase, are interesting since they may help to a better understanding of the structure and function of amylase. Three properties are reported : (1) the affinity of P_1 for polydextran gels. (2) the blocking of P_1 sulfhydryl groups by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). (3) the electrophoretic behavior of P_1 prepared from amylase I and II.

Amylase has some affinity for polydextran gels, possibly by enzyme–substrate type interactions. The enzyme elutes from a G-75 Sephadex column at 2.8 V_0 [9]. P_1 chromatographed on the same column elutes at the same position as the native enzyme (not shown).

Both amylase SH groups react with DTNB in the presence of EDTA [9]. The blocking reaction takes several hours. Trypsin-digested amylase was incubated with DTNB [14]. The stoichiometric liberation of

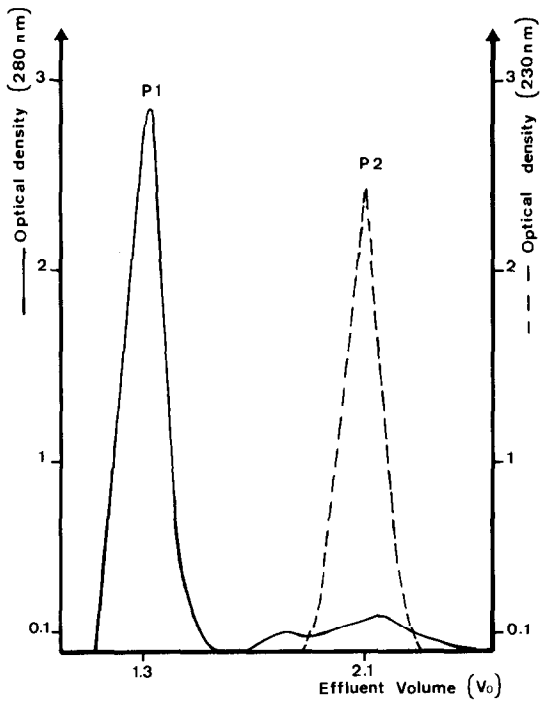

Fig.2. Gel filtration of the trypsin digestion products of amylase. Amylase (1 μmol) was digested (see Materials and methods) and the sample (10 ml) was applied on top of a Biogel P-100 column (2.5 × 100 cm) equilibrated with 5 mM phosphate pH 8. Void volume : 210 ml. Flow rate : 10 ml/hr. Absorbances were simultaneously recorded at 280 nm and at 230 nm. The column was operated at 5°C.

2 TNB ions was instantaneous. Analysis of the reaction mixture was further carried out by gel filtration on Biogel P-100. The stoichiometric blocking of both SH groups was demonstrated.

Amylases I and II have distinct electrophoretic behavior due to different isoelectric points, pH 5.9 and pH 5.4*, respectively [8]. Peptide P_1 (amylase I) and P_1 (amylase II) were analyzed by polyacrylamide gel electrophoresis : the core enzymes differ in their electrophoretic migration as do the native forms (not shown).

* The value for amylase II obtained in our laboratory by electrofocusing differs slightly (i.e. pH 5.25).

In conclusion even after complexion of calcium ions, only limited proteolysis of amylase by trypsin is possible.

Peptide B, because of its blocked N-terminal end may correspond to the N-terminal sequence (N-acetylated) of the amylase chain. Peptide A, because of its C-terminal leucine may correspond to the C-terminal side of amylase. Peptides P_2 can then be linking peptides.

Further studies of these peptides are of special interest in the understanding of amylase structure.

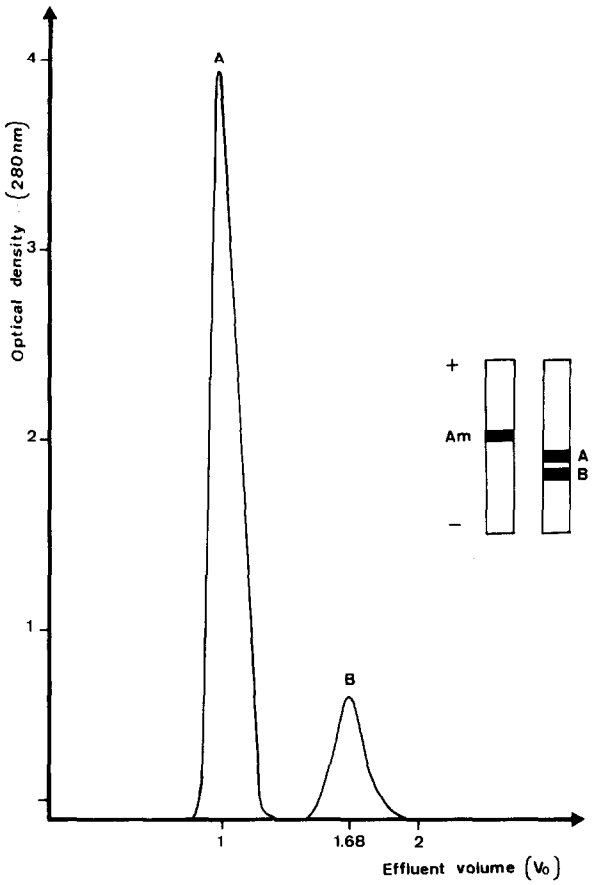

Fig.3. Purification by gel filtration of peptides A and B. P_1 (1 μmol) was dialyzed against water, lyophilized, reduced and carboxymethylated [13]. The sample (5 ml) was applied to a Sephadex G-100 column (2.5 × 100 cm) equilibrated with 4 M urea. Void volume : 160 ml. Flow rate : 15 ml/hr. The column was operated at 20°C in the dark. SDS gel electrophoresis patterns of RCM-P_1 and amylase (Am) are given in the insert. The conditions are as in fig.1.

Acknowledgements

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