

## Partial determination of the amino-acid sequence of porcine pancreatic $\alpha$ -amylase I

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**Summary.** Porcine pancreatic  $\alpha$ -amylase, a single polypeptide chain (470 residues) whose N-terminal amino-acid is acetyl-blocked, is present in two forms, I and II. In this work the amylase I chain was cleaved by cyanogen bromide. Separation of the peptides into seven fractions was obtained by gel filtration ; peptides from fractions 4 and 5 were further purified by repeated gel filtrations and ion-exchange chromatography. CNBr-4 peptide (71 residues) was totally purified from fraction 4. CNBr-5b (62 residues), CNBr-5c (42 residues) and CNBr-5d (38 residues) peptides were isolated from fraction 5 ; sequence analysis was carried out directly on each peptide (Hermodson *et al.*, 1973). Further analysis of the CNBr-4 peptide was achieved by sequencing the three tryptic peptides obtained after digestion. The first eighteen residues of the CNBr-5b peptide were found to be identical to the corresponding sequence in the CNBr-4 peptide. At the present stage, the sequence of three peptides, accounting for about 160 residues, has been analyzed and 115 amino-acids have been positioned, representing 25 p. 100 of the entire sequence of the amylase I chain.

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### Introduction.

Several  $\alpha$ -amylases ( $\alpha$ -1,4 glucan-4-glucano hydrolase, E. C. 3.2.1.1.1) have been isolated from various sources and characterized (Thoma, Spradlin and Dygert, 1971). In mammals,  $\alpha$ -amylase has been purified from the pancreas of hogs (Marchis-Mouren and Pasero, 1967) rats (Vandermeers and Christophe, 1968), rabbits (Malacinski and Rutter, 1969), sheep (Ettalibi, Ben Abdeljlil and Marchis-Mouren, 1975) and mice (Danielsson, Marklund and Stigbrand, 1975) and from human saliva (Stiefel and Keller, 1973). Since  $\alpha$ -amylase is a major constituent of porcine pancreatic juice (Marchis-Mouren, 1965), it is of particular interest in carbohydrate digestion studies. This enzyme is only active on long polyglucan chains ; short oligosaccharides are very poor substrates. Due to the lack of any satisfactory synthetic substrate, not much has been learnt from kinetic studies and very little is known about the mode of action of the enzyme (Thoma, Spradlin and Dygert, 1971).

Porcine amylase is present as two equally active forms (I and II) having about the same molecular weight ( $53\,000 \pm 1\,000$ ; 470 residues) and amino-acid composition, but with distinct isoelectric points (5.95 and 5.25, respectively). The molecule contains 4 disulfide bridges, 2-SH groups and 8 methionines, and the enzyme needs calcium and chloride ions for its activity (Hsiu, Fischer and Stein, 1964; Steer and Levitsky, 1973). Removal of calcium by a chelating agent leads to a conformational change as shown by (i) SH unmasking (Granger *et al.*, 1975) and (ii) enzyme sensitivity to trypsin attack (Granger, Abadie and Marchis-Mouren, 1975). The native enzyme is insensitive to trypsin. Amylase crystals suitable for X-ray crystallography have been obtained (Pierrot *et al.*, 1977). Very recently their 4.5 Å structure has been determined (Pierrot *et al.*, 1979, in progress), thus contributing to knowledge of the mode of action of the enzyme and providing a further comparison of  $\alpha$ -amylase I and II, and sequence studies have begun in our laboratory. In the present work, the amylase (I) chain was cleaved by cyanogen bromide; 4 out of 9 CNBr-peptides were then completely purified and their sequence analyzed.

## Methods.

Porcine pancreatic  $\alpha$ -amylase was purified as previously described (Granger *et al.*, 1975).

**CNBr cleavage conditions** : Lyophilised amylase (120-180 mg) was dissolved in 70 p. 100 formic acid (1 p. 100 final concentration), 180-fold molar excess CNBr was added at zero time and a 2nd addition of CNBr was made 24 hrs later. Cleavage was carried out at 5 °C under nitrogen atmosphere for 40 hrs.

**Filtrations and chromatography** : All columns were operated at 5 °C. The conditions are indicated in the figure legends. Pooled fractions were lyophilised. Urea-containing fractions were dialysed before lyophilisation using appropriate dialysis tubing (Spectrapor 3).

**Citraconylation** : The procedure of Hermodson *et al.* (1973) was used, except that the lyophilised peptide was dissolved in 100 mM ammonium bicarbonate. The citraconylated peptides were separated from citraconate by gel filtration on a Bio-Gel P-2 column equilibrated with 50 mM bicarbonate and finally lyophilised.

**Trypsin digestion** : The citraconylated peptide was dissolved (0.25 p. 100) in 60 mM  $\text{NH}_4$ -bicarbonate (pH 8) and TPCK-Trypsin (Worthington) was added (1 p. 100 of the amount of peptide). Incubation was carried out at 37 °C for 1 hr.

**Reduction and carboxymethylation** : The technique of Crestfield, Moore and Stein (1963) was used.

**Carboxypeptidase digestion** : The technique of Narita, Matsuo and Nakajima (1975) was used.

**Sequencing** : All sequence analyses were carried out using the Beckman Model 890 C sequencer according to the method of Hermodson *et al.* (1973) using the Beckman

0.1 M quadrol program with dual benzene ethyl-acetate wash and polybren as a carrier.

Phenylthiohydantoin derivatives of the amino-acids were identified by high-performance liquid chromatography (Waters) ; after hydrolysis with IH (Smithies *et al.*, 1971) they were identified by the amino-acid autoanalyzer.

## Results.

1) *Purification of the CNBr-4 peptide.* — After CNBr treatment the whole cleavage was immediately filtered through a Bio-Gel P-10 column. As shown on the pattern (fig. 1), five major fractions were obtained : 1, 3, 4, 5 and 7, eluted respectively at 1  $V_0$ , 1.6  $V_0$ , 2  $V_0$ , 2.4  $V_0$  and 3  $V_0$ . Each fraction was pooled and lyophilised. The peptide(s) from fraction 4 was further purified on a Bio-Gel P-6 column (fig. 2 left). A major

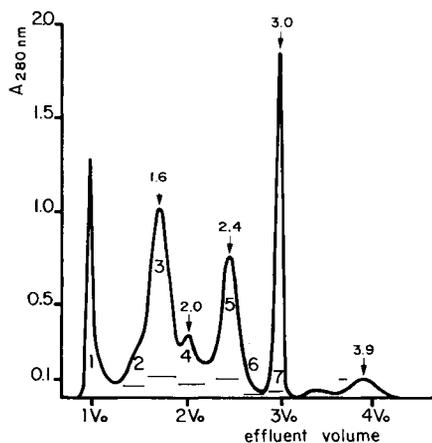


FIG. 1. — Elution profile of the CNBr-peptides of amylase. The column (2.5  $\times$  100 cm) of Bio-Gel P-10 was equilibrated with 7 p. 100 (w/v) formic acid ; flow rate : 20 ml/hr ;  $V_0$  = 177 ml ; sample : 60 mg. The horizontal bars indicate pooled fractions.

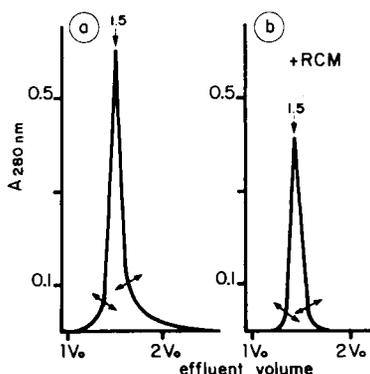


FIG. 2. — Purification of the CNBr-4 peptide by filtration on Bio-Gel P-6. The conditions are the same as in figure 1, except that  $V_0$  = 147 ml ; sample : 10 mg.

component (CNBr-4) eluted at 1.5  $V_0$ . Contaminants on each side of the peak were cut off. The main part of the peak was pooled, lyophilised, reduced and carboxymethylated, and finally filtered in the same conditions (fig. 2 right). As shown on the pattern a single peak eluted at the same position as before (1.5  $V_0$ ). Minor contaminants on each side of the peak were discarded. The purity of the CNBr-4 peptide and its molecular weight were determined from the amino-acid composition (not shown) by the technique of Delaage (1968).

2) *Purification of the CNBr-5a, CNBr-5b, CNBr-5c and CNBr-5d peptides.* — Fraction 5 was filtered through a Bio-Gel P-6 column. Four constituents (5a, 5b, 5c, 5d), eluted respectively at 1.5  $V_0$ , 1.93  $V_0$ , 2.0  $V_0$  and 2.18  $V_0$ , were apparent (fig. 3). Each fraction was pooled separately and further purified.

Fraction 5a was found to contain a single peptide (CNBr-5a) identical to the CNBr-4 peptide, and was joined to fraction 4.

The CNBr-5b peptide was purified from fraction 5b by gel filtration and DEAE-Sephacrose chromatography. First, fraction 5b was filtered through a P-6 column ; as

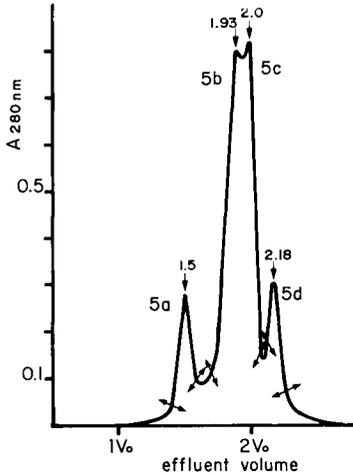


FIG. 3. — Elution profile of the CNBr-peptides of fraction 5. The column ( $2.5 \times 100$  cm) of Bio-Gel P-6 was equilibrated with 7 p. 100 formic acid ; flow rate : 12 ml/hr ;  $V_0 = 148$  ml ; sample : 20 mg.

shown on the pattern (fig. 4a) two peaks were present. The first small peak ( $5b_1$ ) eluted at the same position as the CNBr-4 peptide ( $1.5 V_0$ ) ; the second peak ( $5b_2$ ) containing the CNBr-5b peptide was further purified by passing through a P-6 column in 30 p. 100

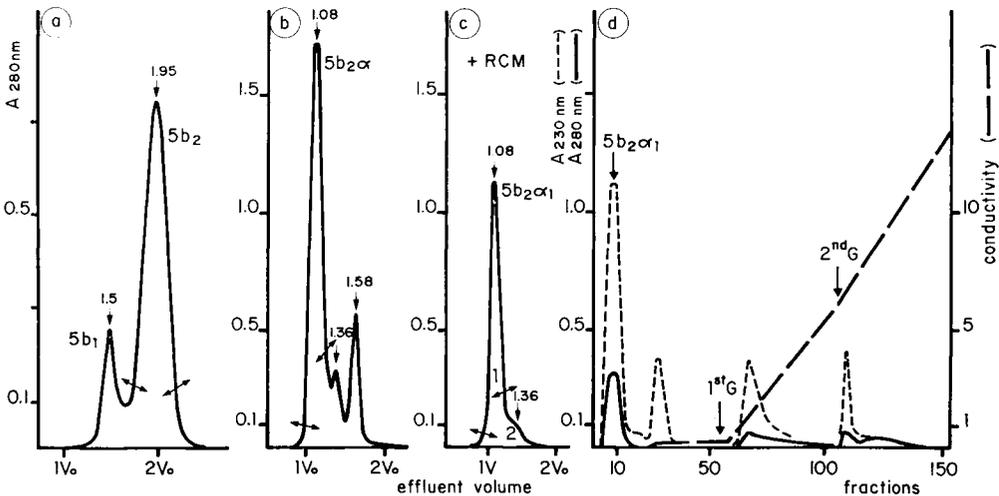


FIG. 4. — Purification of the CNBr-5b peptide. a) The conditions are the same as in figure 3. b) The column ( $1.5 \times 100$  cm) was equilibrated with 30 p. 100 formic acid ; flow rate : 16 ml/hr ;  $V_0 = 52$  ml ; sample : 12 mg. c) The  $5b_2 \alpha$  component was reduced and carboxymethylated, then filtered in the same conditions as in figure 4b. Sample : 8 mg. d) Chromatography of  $5b_2 \alpha_1$  on DEAE-Sephacrose. The column ( $0.9 \times 16$  cm) was equilibrated with 10 mM Tris-HCl buffer (pH 8.5) containing 8 M urea. Flow rate : 36 ml/hr ; sample : 4 mg. The elution was carried out by two NaCl gradients. 1 st gradient : 0-0.2 M ( $2 \times 50$  ml) ; 2nd gradient : 0.2-0.5 M ( $2 \times 50$  ml).

formic acid (fig. 4b). A main peak ( $5b_{2\alpha}$ ) eluted at 1.08  $V_0$  was obtained. This fraction was reduced and carboxymethylated, then filtered in the same conditions as above ; the RCM-peptide ( $5b_{2\alpha 1}$ ) was present at the same position on the pattern (fig. 4c). Final purification of the CNBr-5b peptide was achieved by chromatography through a DEAE-Sephacel column (fig. 4d). The major fraction ( $5b_{2\alpha 1}$ ) was not retained on the column which bound minor constituents. The amino-acid composition of the CNBr-5b peptide was then determined (not shown). It indicated that the peptide was in a pure state.

The CNBr-5c peptide was isolated from fraction 5c by the same procedure as the one used for fraction 5b, except that the DEAE-Sephacel step was unnecessary. A filtration on a P-6 column allowed the separation of 2 minor components,  $5c_1$  (1.58) and  $5c_2$  (1.9), from a major one,  $5c_3$  (2.10). Further purification of the  $5c_3$  fraction was achieved on a P-6 column in 30 p. 100 formic acid (fig. 5b). The main component

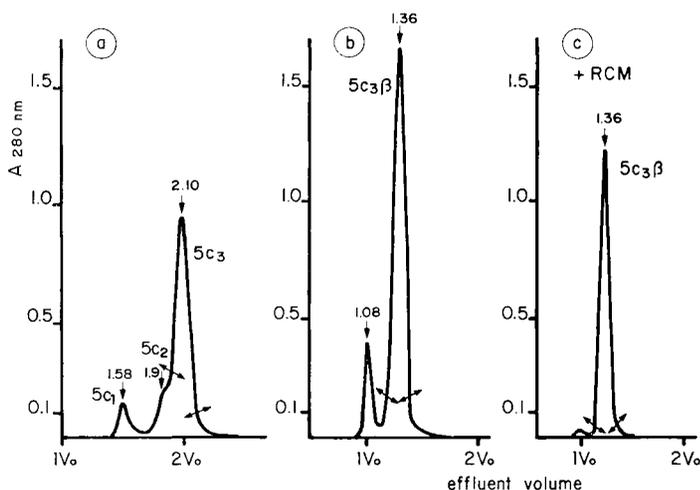


FIG. 5. — Purification of the CNBr-5c peptide on Bio-Gel P-6. a) The column ( $2.5 \times 100$  cm) was equilibrated with 7 p. 100 formic acid ; flow rate : 23 ml/hr ;  $V_0 = 148$  ml ; sample : 15 mg. b) The column ( $1.5 \times 100$  cm) was equilibrated with 30 p. 100 formic acid ; flow rate : 15 ml/hr ;  $V_0 = 52$  ml ; sample : 10 mg. c) The  $5c_3 \beta$  peptide was reduced and carboxymethylated, then filtered in the same conditions as in figure 5b.

( $5c_{3\beta}$ ) was next reduced and carboxymethylated, then filtered through the same column ; a single component ( $5c_{3\beta}$ ), was obtained at the same position (1.36). The amino-acid composition of the CNBr-5c peptide was then determined (not shown) ; it indicated that that peptide was in a pure state.

The CNBr-5d peptide was isolated by filtration of the 5d fraction on a Bio-Gel P-6 column. A single component,  $5d_2$  (1.44) was present (fig. 6a). This fraction was next reduced and carboxymethylated, and finally filtrated on the same column. An identical pattern was obtained (fig. 6b). The purity of this fraction, named CNBr-5d, was assessed by its aminoacid composition (not shown).

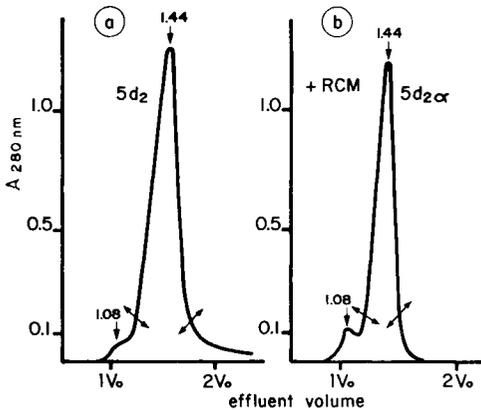
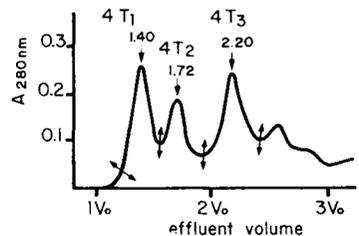


FIG. 6. — Purification of the CNBr-5d peptide on Bio-Gel P-6. a) The conditions are the same as in figure 5b. b) The 5d<sub>2</sub> peptide was reduced and carboxymethylated, then filtered in the same conditions as in figure 5b. Sample : 10 mg.

3) *Sequence studies.* — The sequence determination of the four purified peptides was carried out by the degradation technique of Edman and Begg, modified by Hermodson *et al.* (1973) (see methods).

a) *CNBr-4 peptide* : The sequence of the chain till position 40 (Ile) was directly determined by the use of the sequencer (fig. 8). In order to sequence the remaining part of the molecule the peptide was citraconylated and then digested with trypsin. Tryptic peptides obtained by cleavage at the Arg level were separated by gel filtration on Bio-Gel P-6 (fig. 7). As expected, only three peptides (4-T1, 4-T2 and 4-T3) were obtained

FIG. 7. — Purification of the tryptic peptides of the CNBr-4 peptide digest by filtration on Bio-Gel P-6. The conditions are the same as in figure 5b ; sample : 5 mg.



since the Arg (25)-Pro(26) bond was not attacked by trypsin. The sequence of the 4-T1 peptide corresponded to the N-terminal moiety (1-49) of the entire CNBr-4 peptide. The 4-T3 peptide containing 1 mole per mole of homoserine, thus occupied the C-terminal position (65-71). The 4-T2 peptide was then in the intermediate position (50-64) of the CNBr-4 peptide sequence. The ordering of the entire sequence of the 4-T2 peptide (15 residues) was obtained by the use of the sequencer.

b) *CNBr-5b peptide* : The sequence of the first eighteen residues was found to be identical to the one of the CNBr-4 peptide. This peptide, whose molecular weight is about 6 400, could be due to uncontrolled and partial cleavage of either the CNBr-4 peptide or the amylase chain.

c) *CNBr-5c peptide* : The sequence of the chain till position 31 (Ala) was determined directly by using the sequencer (fig. 9). Partial ordering of the C-terminal part of the molecule was obtained by carboxypeptidase digestion.



from fraction 5 have been totally purified by repeated gel filtrations and, in the case of the CNBr-5b, by additional ion-exchange chromatography.

The amino-acid order of the major part of all the peptides has been determined by sequence analysis using the entire peptides. Surprisingly, the N-terminal sequence (18 residues) of the CNBr-5b peptide was found to be identical to that of the CNBr-4. The CNBr-5b peptide might then originate from the CNBr-4 peptide by molecule shortening due to the acidic medium. However, this hypothesis is not consistent with the presence of homoserine, as indicated by the amino-acid composition of this peptide. Further investigations are thus necessary. Subsequent cleavage of the CNBr-4 peptide was carried out to determine the sequence of the C-terminal part of the molecule. The resulting fragments were purified and arranged in the chain. However, the complete sequence analysis of one of these peptides has not yet been achieved. In the case of the CNBr-5c and CNBr-5d peptides, subsequent enzymatic cleavages are underway to determine the still missing C-terminal sequences. In conclusion, the complete purification of four peptides is presented ; the purification of the other five or six CNBr-peptides from the indicated fractions (1, 2, 6, 7) is nearly achieved. The sequence of three peptides, accounting for about 160 amino-acid residues, has been analyzed and 115 amino-acids have been positioned, representing 25 p. 100 of the amylase chain. When the complete primary structure is obtained, amylase I and II can be compared at the peptide level. Knowledge of the entire sequence would also be valuable to crystallographers for interpreting the 3 Å X-ray data which are now being measured.

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**Résumé.** L' $\alpha$ -amylase pancréatique, une seule chaîne peptidique longue de 470 résidus dont l'extrémité est N-acétylée, est présente sous 2 formes I et II. Dans le présent travail la molécule d'amylase a été clivée par le bromure de cyanogène. La séparation des peptides en sept fractions a été obtenue par tamisage moléculaire. Les peptides des fractions 4 et 5 ont été finalement purifiés par filtrations répétées sur colonne de gel de polyacrylamide et chromatographie d'échange d'ions. Le peptide CNBr-4 (70 résidus) a été obtenu à l'état pur à partir de la fraction 4. Les peptides CNBr-5b (62 résidus), CNBr-5c (42 résidus) et CNBr-5d (38 résidus) ont été isolés de la fraction 5. La séquence de chaque peptide a été partiellement déterminée par analyse directe au séquenceur (Hermodson *et al.*, 1973). Une détermination plus complète de la séquence du peptide CNBr-4 a été réalisée à partir des 3 peptides obtenus après digestion trypsique. Les 18 premiers résidus du peptide CNBr-5b sont identiques à ceux du CNBr-4. Actuellement la séquence de 3 peptides représentant 160 résidus est en cours d'élucidation : 115 amino-acides représentant 25 p. 100 de la séquence de la chaîne d'amylase I ont été positionnés.

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