ON A CHYMOTRYPSIN C PURIFIED FROM AUTOLYZED PORCINE PANCREAS

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SUMMARY. A chymotrypsin C was purified from autolyzed porcine pancreas and found to differ from the π enzyme only by the loss of the four C-terminal residues of the A-chain.

Porcine pancreas and pancreatic juice have recently been shown (1) to contain 3 ChTg** : ChTg A (2) and B (3) whose amino acid composition and general properties are similar to those of the corresponding bovine zymogens, and ChTg C (4) with a distinctly different composition and a somewhat higher molecular weight (3, 4). When compared to the ChTg of the A and B types, porcine ChTg C also possesses an apparently characteristic double deletion at position 12 and 13 (1, 5) and the affinity of the resulting enzyme (ChT C) for leucine bond is higher than in the case of ChT A and B (4, 6). All these features are shared by the ATEE-splitting subunit II of trimeric bovine procarboxypeptidase A (5), which is probably the ChTg C of cattle pancreas (1).

ChTg A, B and C are known to be activated by the tryptic cleavage of the first "basic" bond of their N-terminal sequence. Activation of the first two zymogens is sometimes followed by a limited autolysis of the primary chymotrypsin, or its runs parallel with a limited chymotryptic attack of the zymogen. However, the number of residues split off during both processes does not

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** The following abbreviations are used : ChTg, chymotrypsinogen ; ChT, chymotrypsin ; ATEE, acetyl-L-tyrosine ethylester.

exceed 4 or 5, with the consequence that the molecular weight of the most degraded form so far known of active chymotrypsin (ChT A_α and B_α) does not differ from that of the respective zymogens by more than 600. In contrast, Folk and Schirmer (4), who ascribed to porcine ChTg C a molecular weight of 31,800 daltons, found for a ChT C isolated from autolyzed pancreas a value not exceeding 23,000. If real, this large difference would mean that the activation of ChTg C implies the loss of a sizeable portion of the zymogen molecule, as it is the case for instance for procarboxypeptidase A (7).

In a previous report from this Laboratory (1, 3) pure porcine ChTg C was shown by a number of independant techniques to have a molecular weight of about 29,000 daltons. Tryptic activation at 0° leads to ChT C_π which quickly autolyzes at the level of bonds Leu_{10}-Ser_{11} and Asn_9-Leu_{10} with the concomitant loss of the four last residues of the A chain. However, previous autolysis of pancreas under uncontrolled conditions might induce more extensive degradations. For this reason, pure samples of ChT C were purified from autolyzed pancreas* by a modification of Folk's technique and some of their molecular properties (N-terminal residues and molecular weight) were carefully investigated. No evidence was obtained that they significantly differed from those of ChT C_π.

Autolysis of porcine pancreas was performed as indicated earlier (4) and the acetone powder (100 g) was extracted with water. The extract was clarified by centrifugation and precipitated at pH 7.0-7.2 and 4° with 0.6 saturated ammonium sulfate (390 g/l). All subsequent operations were carried out at 4°. The precipitate was dissolved in water (50 ml) and dialyzed overnight against a 10 mM Tris-acetate buffer pH 6.0. The clarified solution was charged into a DEAE-cellulose column equilibrated with the above buffer. A linear NaCl concentration gradient (0 → 0.4 M) was applied with the results indicated by fig. 1a.

The fractions composing the more anionic peak on the right

* This hypothetical chymotrypsin will provisionally be designated ChT C_a.

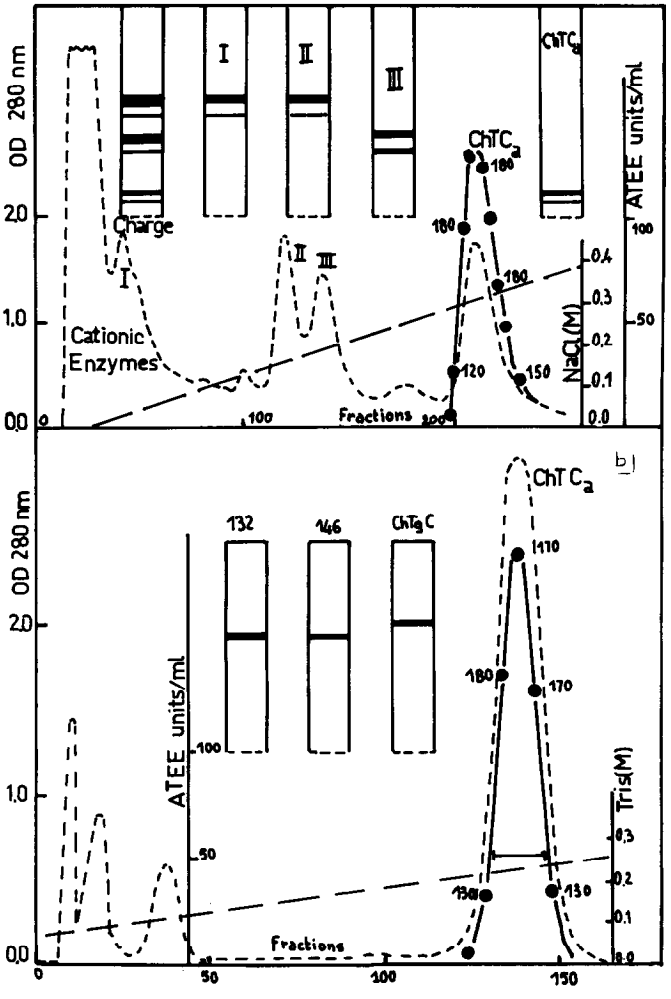

Fig. 1 Purification of Chymotrypsin C from autolyzed porcine pancreas.

Fig. 1a : Chromatography of the aqueous extract (7,1 g of proteins ; 525,000 ATEE units) in a 4 x 40 cm DEAE-cellulose (Whatman DE 11) column equilibrated with a 10 mM Tris-acetate buffer pH 6.0 and eluted at the same pH by a linear NaCl concentration gradient from 0 to 0.4 M (chamber volume, 4 l). Solid line : chymotryptic activity measured against ATEE. Interrupted line : protein content of the fractions determined by spectrophotometry at 280 nm. Fraction volumes, 30 ml. Flow rate : 150 ml/h. The specific activity (number of ATEE units per mg protein) of the fractions are indicated by numbers along the ChT C peak. The disc electrophoresis assays whose diagrams are reproduced in the figure were carried out at pH 8.6 with a 7.5 % polyacrylamide gel.

Fig. 1b : Chromatography of the active fractions (fig. 1a) on a 2.5 x 60 cm CM-Sephadex C 50 (Pharmacia, Uppsala) column equilibrated with a 50 mM Tris-acetate buffer pH 6.0 and eluted by a linear increase of the buffer concentration from 0.05 to 0.3 M (chamber volume : 920 ml). Volume of fractions, 10 ml. Flow rate : 30 ml/h. Aliquots of fractions 132 and 146 and of a solution of pure ChTg C were submitted to disc electrophoresis at pH 8.6 in a 15 % polyacrylamide gel. Other symbols are the same as in fig. 1a.

of the diagram were active against ATEE. Disc electrophoresis at pH 8.6 showed that they contained a main component and at least one impurity. UV spectrophotometry revealed that they were also contaminated by appreciable amounts of nucleic acids.

The fractions were pooled and precipitated in 0.8 saturated ammonium sulfate. After dialysis of the precipitate against a 50 mM Tris-acetate buffer pH 6.0, the solution was submitted to chromatography on CM-Sephadex. Fig. 1b shows that ChT C_a emerges from the column in a substantially pure form for a buffer concentration of about 0.2 M. The more anionic protein contaminants detected by disc electrophoresis in fig. 1a and the nucleic acids are eluted at a lower molarity. The fractions indicated in fig. 1b

Table I Molecular weight determination of ChT C_a

Technique	Solvent	Concentration of solutions. (mg/ml)	Estimated molecular weight.
Yphantis	0.1 M Tris buffer (pH 6.0)	0.35	32,690*
Yphantis	0.8 M Tris buffer (pH 6.0)	0.35	28,180**
Yphantis	10 mM HCl (pH 2.0)	0.25	28,900*
Archibald (31,410 RPM)	0.3 M Tris buffer (pH 6.0)	3.9	28,120* ± 470
Archibald (21,740 RPM)	0.3 M Tris buffer (pH 6.0)	3.9	28,120* ± 470
Chemical analysis (8)	-	-	27,500
Electrophoresis (9)	-	-	28,000-29,000

* The adopted value for the partial specific volume (0.73) was derived from the amino acid composition (Table II).

** This sample was a kind gift of Dr. J.E. Folk (Bethesda).

by an horizontal bar were pooled, dialyzed against water and lyophilized. The yield was 500 mg of pure ChT C_a per 100 g of acetone powder.

The original purification procedure of Folk and Schirmer (4) was applied to a sample of acetone powder prepared in this Laboratory. The same procedure was applied with similar results and the procedure described here was also applied to an acetone powder sample kindly sent by Dr. J.E. Folk. All the ChT C_a thus obtained had the same behavior during chromatography and disc electrophoresis.

Molecular weight determinations of the purified enzyme were attempted in several ways. Exactly the same elution volume was observed when 6 mg/ml solutions of ChT C_a and ChTg C in 50 mM Tris-acetate buffer (pH 6.0) containing 0.4 M NaCl were filtrered through a 1 x 195 cm Sephadex G 100 column. Moreover, ultracentrifugation assays by the Yphantis and Archibald techniques were carried out at various concentrations, ionic strengths and pH. As shown by Table I, the values obtained were consistent with a molecular weight of 28,000-29,000 daltons. Similar values were derived from the amino acid composition of the protein by the mode of calculation reported by Delaage (8) and from electrophoresis experiments in the presence of sodium dodecyl-sulfate (9).

Table II gives the amino acid composition of the protein (10). A substantial agreement is seen to exist with the results previously reported by Folk and Schirmer (4), after recalculation to a molecular weight value of 28,000 daltons. The composition of ChTg C is also included in the Table for comparison. Within experimental errors, a few residues only appear to be missing in the enzyme when compared to the zymogen.

Finally, a sample of ChT C_π was carefully prepared (3) by activation of pure ChTg C at 4° in the presence of 0.2 M β-phenylpropionate in order to avoid any undesirable autolysis and/or chymotryptic attack. The N and C-terminal residues of this sample were compared to those of ChT C_a. Only DNP-Val and the peptide DNP-Val-Val could be identified after treatment of the proteins by dinitrofluorobenzene, indicating that bond Arg_{13}-Val_{14} was the single one to be split in both cases during activation. The short A chain of ChT C_a was also isolated after performic oxidation and found to

Table II Amino acid composition of ChT C_a

	Number of residues in ChT C_a			ChTg C (taken from ref (3))	Difference ChTg C - ChT C_a
	Experimental values	Nearest integral numbers	Recalculated from ref (4)		
Ala	13.5	13-14	14	15	1-2
Arg	8.1	8	9	9	1
Asn	25.3	25	26	25	0
Cys*	10.0	10	9	10	0
Glx	25.4	25	25	26	1
Gly	27.2	27	28	26-27	0
His	6.4	6	6	6	0
Ile	14.1	14	13-14	14	0
Leu	21.3	21	22	22	1
Lys	7.4	7	8	7	0
Met	0.96	1	1	1	0
Phe	4.1	4	5	4	0
Pro	15.1	15	14-15	13-14	1-2
Ser**	21.7	22	24	22-23	0-1
Thr**	15.9	16	17	17	1
Trp***	12.0	12	10	12	0
Tyr	6.05	6	7	6	0
Val	22.9	23	22-23	23	0
TOTAL	-	255-256	260-263	258-261	6-9

* As cysteic acid after performic oxidation of the protein.
** After extrapolation to zero time hydrolysis.
*** By spectrophotometry.

contain 9 residues instead of 13 in ChT C_π. Residues Arg_{13}, Ala_{12}, Ser_{11} and Leu_{10} were missing, as in ChT C_δ normally arising during autolysis of ChT C_π.

Native ChTg C is not attacked by a mixture of carboxypeptidases A and B (4). Consequently, the S-carboxymethyl derivatives of ChT C_a, ChT C_π and ChTg C were prepared and their C-

terminal residues were compared. DFP-treated carboxypeptidase A liberated leucine, glutamine and isoleucine from the three proteins at a rate suggesting the following sequence (Ile,Gln)-Leu-OH. The C-terminal position of leucine was confirmed in each case by hydrogene-tritium exchange (11). The sequence (Ile,Gln)-Leu clearly arises from the C-terminal region of ChTg C. In fact, carboxypeptidase A is unable to detach the additional C-terminal Arg_{13} in ChT C_π and also Asn_9 in ChT C_a because of the presence of proline in position 8. A relatively weak incorporation of tritium observed into glutamine might indicate either the presence of γ-glutamyl linkages in ChT C_a (12) or, more probably a partial autolysis of the C-terminal leucine.

The experimental evidence reported above proves that, in spite of the fairly drastic conditions prevaling during autolysis of porcine pancreas at room temperature, ChT C_a does not essentially differ from ChT C_π, except for the loss of four residues. The molecule, therefore, should be considered as exceptionally resistant to proteolytic attack. The cleavages taking place in ChTg A and B molecules, leading to the formation of the chymotrypsins of the α type do not even occur in the case of ChT C.

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