Affinity purification of chicken pancreas proteinases and their N-terminal amino-acid sequences*

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Despite their importance for the nutritionist, very little is known about the nature of pancreatic enzymes of avian species. Ryan [1] purified trypsin from the turkey and chymotrypsin from the chicken pancreas. Hartley et al. [2] using extraction at alkaline pH and trasylol-Sepharose affinity chromatography for purification, partially characterized a cationic trypsin from the ostrich pancreas. More recently, Smith et al. [3] isolated and partially characterized trypsinogen, and multiple forms of chymotrypsinogen and trypsin from the pancreas of the same species.

This paper presents the results on the simultaneous purification of trypsin, chymotrypsin and elastase by serial affinity column chromatography on *Cucurbita maxima* trypsin inhibitor (CMTI I), basic pancreatic trypsin inhibitor (BPTI) and soybean trypsin inhibitor (STI) bound to Sepharose 4B. The N-terminal sequences of the purified enzymes are also presented.

Extraction and activation of proteolytic enzymes. Pancreatic tissues removed from freshly killed 4 week old chicken were cut into small pieces and homogenized for 5 min with 3 vol. of 0.5 M $CaCl_2$ containing 0.2% sodium azide. The most favorable conditions for simultaneous extraction and activation of trypsin, chymotrypsin and elastase were found to be: incubation of the homogenate at pH 7.5 for 9 h at room temperature. Under these conditions trypsin and chymotrypsin reached maximum activity whereas elastase had only 65% of its maximal activity.

Separation of enzymes by affinity chromatography. Clarified extract from chicken pancreas was adjusted to pH 8.0 and loaded sequentially into three affinity columns prepared by coupling CMTI I, BPTI and STI to cyanogenbromide-activated Sepharose 4B [4]. The size of the column was so adjusted that the binding capacity of each exceeded by 40% the amount of enzyme in the extract. The first column used (CMTI I-Sepharose), was washed with 0.2 M Tris/HCl buffer, pH 8.0, and the flow-through fractions kept for the next step. The column was further washed with 0.2 M Tris/HCl, pH 8.0, containing 1 M NaCl until the absorption fell below 0.01. Bound trypsin was eluted with 0.001 M HCl.

The flow-through fraction from the CMTI I column was immediately applied to a BPTI affinity column. A similar procedure as described above for trypsin isolation was followed to wash out and elute chymotrypsin from the column. The non-adsorbed material from the BPTI column was loaded onto an STI

column, washed out at pH 8.0 and the elastase eluted at pH 3.0.

In our studies we were able to separate highly purified trypsin, chymotrypsin and elastase by taking advantage of both the specificity of the inhibitors used and certain properties of chicken proteolytic enzymes. CMTI I has been shown to bind only trypsin [5] and therefore was used in the first affinity column. BPTI, notwithstanding its rather broad inhibitory specificity, does not inhibit pancreatic elastase [6]. Therefore, only chymotrypsin was bound on the BPTI affinity column. In turn, STI is known to inhibit trypsin, chymotrypsin but not pancreatic elastase [6], however, we found that chicken pancreatic elastase was inhibited by STI. Because of this trait of the latter enzyme, we used an STI-affinity column for our final isolation step. We found that the sequential CMTI/BPTI/STI affinity chromatography was the fastest procedure and gave the best results in simultaneous isolation of trypsin, chymotrypsin and elastase from the chicken pancreas, with the yield exceeding 60% (Table 1).

Ion exchange chromatography of the enzyme preparations. Electrophoretic analysis of the separated enzymes revealed that all of them consisted of two or three active forms. The trypsin preparation consisted of one predominant anionic form and two cationic forms. In the chymotrypsin preparation three active components were found. The elastase preparation showed two distinct forms migrating close to each other.

To separate individual components, the enzyme preparations were subjected to ion exchange chromatography on Mono Q column (Pharmacia, Uppsala, Sweden) (Fig. 1). In the elution profiles of trypsin, 3 main peaks were observed. The first peak (T1) was found to be an anionic form of trypsin, whereas the other

two (T2 and T3) were cationic forms. The chymotrypsin preparation was resolved into 3 main peaks, of which two appeared to contain more than one enzyme form and only peak C appeared on electrophoresis as a single band. The elution of elastase gave two well separated peaks (E1 and E2) with elastolytic activity. SDS-PAGE under reducing conditions revealed that, except for preparation T3, all separated forms of the enzymes after treatment with diisopropyl fluorophosphate (DFP) migrated as single bands of 23.5, 24.0 and 25.0 kDa for trypsin, chymotrypsin and elastase, respectively. Trypsin T3 showed a main band of 16.5 kDa and one minor band of about 7 kDa suggesting that it consisted of a two-chain molecule.

N-Terminal amino-acid sequence. For each form of trypsin, 23 to 24 residues were recovered (Fig. 2). The anionic form (T1) and one cationic form (T2) gave one N-terminal sequence each, whereas two sequences were obtained from T3, one of them identical to that of T2 form. For chymotrypsin C, two N-terminal amino-acid sequences were read, with 16 and 24 residues, respectively (Fig. 3). It is worth to note that, in our electrophoresis analysis of the M_m of chymotrypsin C, the shorter chain was not taken into account and therefore 1.7 kDa corresponding to the latter fragment should be added, thus making our estimation of M_m of chymotrypsin C equal to 25.7 kDa, like that reported by Ryan *et al.* (7). No differences in the N-terminal amino-acid sequences were detected between the two forms of elastase up to the 23rd residue (Fig. 4).

The N-terminal amino-acid sequence analysis showed some homologies of chicken trypsin and chymotrypsin with the bovine enzymes, and of chicken elastase with the porcine enzyme. For trypsin, the percentage of identity between the bovine enzyme and the chicken

Table 1

Recovery of proteolytic enzymes from 20 g of chicken pancreas

Enzyme	Total activity (in units*)		
	In extract	In lyophilized preparation	Yield (%)
Trypsin	20680	15100	73
Chymotrypsin	3410000	2560000	75
Elastase	27100	16800	62

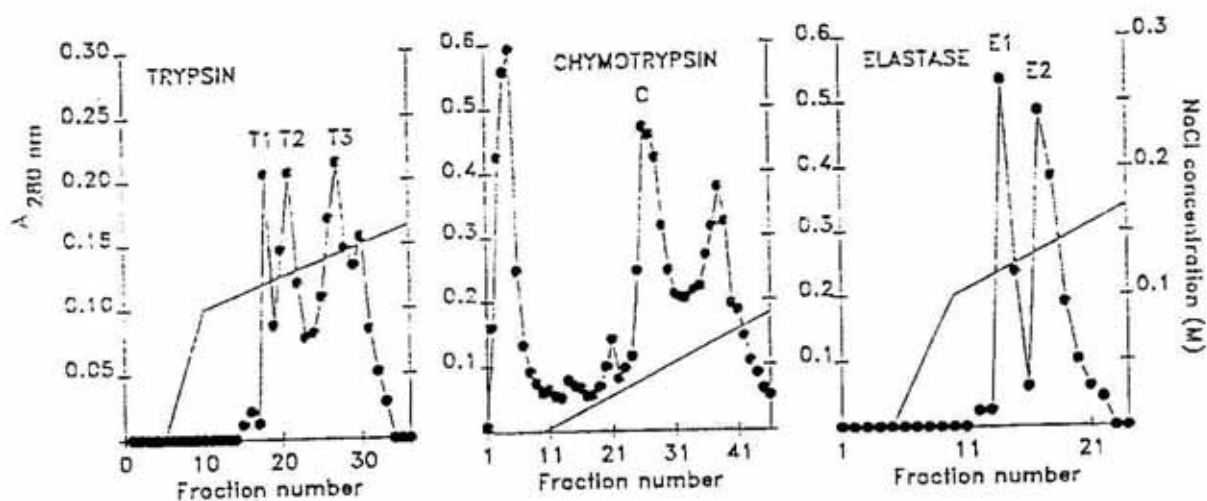*1 unit = increase by 0.01/min of the absorbance at 410 nm under conditions used.

Fig. 1. F.p.l.c. ion exchange chromatography of trypsin, chymotrypsin and elastase on Mono S column (Pharmacia, Uppsala) at pH 3.0, 5.0 and 4.7, respectively.

enzyme in the N-terminal portion was 74 and 83% for the anionic and cationic form, respectively. The chicken cationic trypsin compared favorably (over 90% identity) with the ostrich trypsin. The cationic form T3 gave 2 sequences, one of them identical to the N-terminal of T2 and the second representing the N-terminal sequence of the second chain of the molecule. This second sequence presented 59% homo-

logy with the portion of bovine α-trypsin from Ser130 to Tyr151. Accordingly, we consider that T2 corresponds to the β-form and T3 to the α-form of the cationic trypsin.

Chymotrypsin C also gave two N-terminal sequences. The shorter chain corresponded to the first 15 amino-acid residues of the bovine chymotrypsinogen, showing a 50% homology. It is worth noting that the chicken chymotryp-

Fig. 2. N-Terminal amino-acid sequence of the chicken trypsin T1, T2 and T3 as compared to bovine trypsin and ostrich trypsin.

Fig. 3. N-Terminal amino-acid sequence of the chicken chymotrypsin (C) as compared to bovine chymotrypsin.

Fig. 4. N-Terminal amino-acid sequence of the chicken elastases as compared to porcine elastase.

sin differs from the bovine enzyme by a tetra-peptide (SLPS) which extends beyond cysteine. The longer chain corresponds to the segment Ile16 to Glu34 of the bovine chymotrypsinogen, showing a 79% homology. These date indicate that we have purified and partially sequenced the π-like form of chicken chymotrypsin.

The N-terminal sequence of the chicken elastases showed about 75% homology with the porcine elastase.

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