The Chymotrypsinogens and Procarboxypeptidases of Chick Pancreas*

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SUMMARY

The soluble proteins of chick pancreas zymogen granules were chromatographed on a DEAE-cellulose column. Enzymes and zymogens were eluted with a concentration gradient of phosphate buffer pH 8.0 (5 to 100 mM) in the following order: chymotrypsinogen 1 (void volume), trypsinogen 1, trypsinogen 2, carboxypeptidase A, procarboxypeptidase B, lipase, procarboxypeptidase A, chymotrypsinogen 2, chymotrypsinogen 3 (coinciding with a minor procarboxypeptidase A peak) and, finally, amylase. Procarboxypeptidases A and B were identified by their specificities towards peptide substrates with terminal aromatic and basic amino acids, respectively. Chymotrypsinogens 1 and 2 on activation produced chymotrypsins with different specificities towards N-acetyl-L-tryptophan ethyl ester and N-benzoyl-L-leucine ethyl ester in the presence of methanol. The relative activities on these substrates indicate that chymotrypsinogen 1 and 2 correspond with mammalian chymotrypsinogens B and A, respectively. Preliminary specificity studies indicate that chymotrypsinogen 3 may correspond with mammalian chymotrypsinogen C.

Since the exportable proteins of the exocrine pancreas are of considerable interest from an evolutionary point of view, the composition of pancreatic juice or zymogen granule contents has been extensively studied in many mammals and some other vertebrate species (1-4). Surprisingly little is known, however, about the protein components of avian zymogen granules. A few of the exportable enzymes of chick pancreas, namely amylase (5), a cationic chymotrypsin (6, 7), and an endonuclease (8, 9), have been partially or completely purified. Some studies have also been carried out on enzymes in crude extracts of chick pancreas (10-13), but no comprehensive survey of chick pancreas enzymes has hitherto been undertaken. Since birds occupy a unique position in the evolutionary scheme, it seemed of interest to obtain more detailed information on the nature of the exportable enzymes of the chick pancreas. Such a study would also provide a basis for investigations of mechanisms of regulation of the biosynthesis of chick pancreas zymogen granule proteins during development (11, 14).

Zymogen granules were chosen as a starting material since they contain the exportable proteins in a concentrated form and their protein composition is similar to that of pancreatic juice (15, 16). Moreover, preparation of zymogen granules presents less technical difficulties than the collection of pancreatic juice. In the investigation outlined below, zymogen granule contents were chromatographed on a DEAE-cellulose column and some of the properties of the proteolytic zymogen fractions were studied. A preliminary report of some of the findings has been published (17).

MATERIALS AND METHODS

Materials

The following compounds were purchased from Cyclo Chemical Company: N-acetyl-L-tryptophan ethyl ester, N-benzoyl-L-leucine ethyl ester, N-carbobenzoxyglycyl-L-phenylalanine, hippuryl-L-arginine, hippuryl-L-lysine, hippuryl-L-phenyllactic acid, and hippuryl-L-phenylalanine. p-Toluene sulfonyl-L-arginine methyl ester and sodium taurocholate were purchased from Mann. N-Acetyl-L-tyrosine ethyl ester and N-benzoyl-L-tyrosine ethyl ester were products of Yeda, Rehovot, Israel. Gum arabic, olive oil (highly refined low acidity) and takadiastase (cw-amylase from Aspergillus oryzae, crude type IV A) were obtained from Sigma. Trypsin, trichry crystallized, salt free, and crystalline soybean trypsin inhibitor were bought from Worthington Biochemical Corporation. Whatman DE-52 microgranular DEAE-cellulose was obtained from Balston, Maidstone, England.

Preparation of Zymogen Granules

Pancreases were removed from five to seven White Leghorn X New Hampshire chickens aged from 6 to 12 weeks, which had fasted for 24 hours. The pancreases were washed several times with 0.25 M sucrose containing 0.01% soybean trypsin inhibitor. They were then placed in 1.5 volumes of 0.25 M sucrose at 0° and all subsequent operations were carried out at 0–4°. After being minced finely with scissors the pancreases were homogenized by 30 strokes of a loose Potter-Elvehjem homogenizer, 90-ml capacity, 0.5-mm clearance at 200 rpm. The homogenate was filtered through 2-mm plastic gauze and the residue on the gauze was homogenized once more with 2.5 volumes of 0.25 M sucrose using 10 strokes of a loose homogenizer. The homogenized resi-
Due was filtered through plastic gauze. Homogenates from the first and second homogenization were pooled and centrifuged for 5 min at 250 x g to sediment nuclei and cell debris. The pellet was resuspended in 0.5 volumes of sucrose and centrifuged for 5 min at 250 x g. The supernatants of the first and second centrifugation were pooled and centrifuged for 10 min at 2000 x g. The pellet consisted mainly of zymogen granules with some contamination by other structures such as mitochondria.

Lysis of Zymogen Granules

The zymogen granule pellet was suspended in 2.5 times its volume of 5 mM phosphate buffer pH 8.0 containing 0.1% Triton X-100. A portion of this lysate was kept without further additions for the estimation of trypsinogen and chymotrypsinogen. Soybean trypsin inhibitor was added to the remainder to give a final concentration of 0.01%. Both portions of the lysate (with and without soybean trypsin inhibitor) were centrifuged for 60 min at 100,000 x g to remove zymogen granule membranes and other insoluble debris. The supernatant was freed from fat and dialyzed overnight against three changes of 5 mM phosphate buffer pH 8.0. The dialyzed solution will be referred to as "zymogen granule extract" in the rest of the paper.

Column Chromatography

Microgranular DEAE-cellulose (Whatman DE-52) was suspended in 0.06 M phosphate buffer pH 8.0 and after removal of fines was poured into a column (1.0 x 70 cm). The column was equilibrated with 5 mM phosphate buffer, pH 8.0.

The chromatographic procedure was based on previously described methods (16, 18). Zymogen granule extract containing 80 to 200 mg of protein was applied to the column. Elution was performed at 4°C at a flow rate of 15 to 20 ml per hour and fractions of 5 to 6 ml were collected. After collection of the void volume several protein peaks were eluted with the equilibrating buffer. Then a gentle linear concentration gradient of phosphate buffer, pH 8.0, containing 0.1 M NaCl was added to the reaction mixture because it formed a sediment with the phosphate in the fractions. Activity without CaCl2 was 8 to 15% less than activity with CaCl2.

Enzyme Activities

Amylase—Activity was determined according to the method of Bernfeld (19), a unit being defined as the amount that in 3 min at 30°C catalyzes the appearance of reducing groups equivalent to 1 mg of maltose hydrate.

Lipase—Activity was determined by the method of Marchis-Mouren, Sanda, and Desnuelle (20). No CaCl2 was added to the reaction mixture because it formed a sediment with the phosphate in the fractions. Activity without CaCl2 was 8 to 15% less than activity with CaCl2.

Estimation of Enzyme Activities

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Analytical Methods

Proteolytic Enzymes—Activities were measured with a Cary model 14 Spectrophotometer at 25°C using cuvettes with a 10-mm light path containing 1.0 ml of reaction mixture. A unit is defined as the amount of enzyme which hydrolyzes 1 μmole of substrate per min.

Chymotrypsin assays were performed in 0.025 M Tris-chloride buffer, pH 8.0. Breakdown of N-acetyl-L-tyrosine ethyl ester (1 mM) was measured at 237 nm (21). Hydrolysis of N-acetyl-L-tryptophan ethyl ester (1 mM) was measured at 248 nm (22), the absorbance difference at 100% hydrolysis being 0.25. Hydrolysis of N-benzoyl-L-arginine ethyl ester (1 mM) was measured at 254 nm (22). Breakdown of N-benzoyl-L-lysine ethyl ester (5 x 10^-4 M) was assayed in presence of 2.5% methanol at 254 nm (22, 23).

General carboxypeptidase activity was measured with hippuryl-m-phenyllactic acid, while carboxypeptidase A activity was measured with hippuryl-L-phenylalanin and N-carboxbenzoyl-L-phenylalanine and carboxypeptidase B activity was measured with hippuryl-L-arginine or hippuryl-L-lysine.

Rates of hydrolysis of 1 mM hippuryl-DL-phenylacetic acid or hippuryl-L-phenylalanine in 0.025 M Tris-chloride buffer, pH 8.0, containing 0.3 M NaCl were determined according to the method of Folk and Schirmer (24). Hydrolysis of 1 mM hippuryl-L-arginine or hippuryl-L-lysine was followed in 0.025 M Tris-chloride, pH 8.0, containing 0.1 M NaCl according to the method of Folk et al. (25). The absorbance difference at 254 nm for 100% hydrolysis of all the hippuryl compounds was 0.35.

The activity of carboxypeptidase A on N-carboxbenzoxycly-L-phenylalanine (5 x 10^-4 M) in 0.03 M Tris-chloride buffer, pH 8.0, containing 0.5 M NaCl was measured at 222.5 nm (26).

Trypsin activity on P toluene sulfonyl-L-arginine methyl ester (10^-3 M) was assayed in 0.05 M Tris-chloride buffer, pH 8.0, at 247 nm (23).

Analytical Methods

Protein was determined by measuring absorbance at 280 nm or by the method of Lowry et al. (27) using crystalline bovine plasma albumin as standard. Where the phosphate concentration was above 0.1 M, protein was precipitated with trichloroacetic acid (final concentration 5%) and redissolved in 0.1 M NaOH prior to assay by the Lowry method.
Inorganic phosphate was determined according to the method of Fiske and SubbaRow (28).

RESULTS

Elution Profile of Protein—Eleven major peaks were discernible (Fig. 1C). Three peaks (a, b, c) were eluted with the equilibrating buffer while eight more peaks were eluted after application of the concentration gradient. Chymotrypsinogen 1 was eluted with the void volume peak (a) while trypsinogen 1 approximately coincided with Peak b. Procarboxypeptidase A, procarboxypeptidase B, and chymotrypsinogen 2 coincided with Peaks 1, 2, and 4, respectively. No measured activities were found to correspond with Peaks c, 3, 5, or 6. Chymotrypsinogen 3 and the minor procarboxypeptidase peak coincided with Peak 7 while Peak 8 was obviously composed mainly of amylase.

Trypsinogens—Trypsinogen is a minor component of chicken pancreas zymogen granules, its potential activity (in p-toluenesulfonyl-L-arginine methyl ester units) being only about 5 to 10% that of chymotrypsinogen (in N-acetyl-L-tyrosine ethyl ester units). Two peaks of trypsinogen, a small weakly anionic peak, and a larger more strongly anionic peak were eluted from the column (Fig. 1B).

Procarboxypeptidases and Carboxypeptidase—Four peaks of activity on hippuryl-n-phenyllactic acid were observed: two very small peaks immediately followed by a large peak eluted between 0.025 and 0.03 M phosphate and another small peak which was eluted just before the main peak of amylase by 0.07 M phosphate (Fig. 1). The fractions of the first small peak (CpA) were active without prior activation. These fractions were active on hippuryl-n-phenylalanine and N-carbobenzoxyglycyl-n-phenylalanine, but showed little activity on hippuryl-L-arginine (Fig. 2) indicating that this peak is carboxypeptidase A.

Fractions of the second small peak (CpB) were inactive without activation and rapidly hydrolyzed hippuryl-L-arginine or hippuryl-L-lysine, but had little activity towards hippuryl-n-phenylalanine (Fig. 2). The preferential specificity for substrates with terminal basic amino acids indicates that this peak is procarboxypeptidase B.

The major peak of activity on hippuryl-n-phenylalanine (CpP4) required activation and was specific for hippuryl-L-phenylalanine and N-carbobenzoxyglycyl-L-phenylalanine, showing little activity on hippuryl-L-arginine (Fig. 2). It was therefore concluded that this peak consists of procarboxypeptidase A.

The procarboxypeptidase peak in the amylase region (PcpA') coincided with the peak of chymotrypsinogen 3. After activation the zymogen hydrolyzed hippuryl-L-phenylalanine and N-carbobenzoxyglycyl-L-phenylalanine, but had no detectable activity on hippuryl-L-arginine (Fig. 2), indicating that the procarboxypeptidase in this peak is of the A type.

Chymotrypsinogens—Three peaks of chymotrypsinogen were observed. A major cationic peak (chymotrypsinogen 1) was...
Chyomotrypsins 1 and 2 were eluted by the gradient (Fig. 1B). About half of the activity on N-acetyl-L-lysine ethyl ester was found in all cases in the chymotrypsinogen 2 peak and most of the residual activity in the chymotrypsinogen 1 peak. Activities recovered from the chymotrypsinogen 3 peak varied considerably (from 0.2% to 10% of the total N-acetyl-L-lysine ethyl ester activity) from one zymogen granule extract to the next. Measurements of the activity of chymotrypsins 1 and 2 on different substrates in presence of varying concentrations of methanol revealed that the two peaks of chymotrypsinogen have different specificities (Table I). Chymotrypsin 2 is more active than chymotrypsin 1 on N-benzoyl-L-lysine ethyl ester, N-acetyl-L-lysine ethyl ester, and N-benzoyl-L-lysine ethyl ester.

Moreover, the activity of chymotrypsin 1 on N-acetyl-L-lysine ethyl ester is greatly reduced in presence of 30% methanol, while chymotrypsin 2 retains much of its original activity. Both chymotrypsinogens 1 and 2 show very little activity on N-benzoyl-L-lysine ethyl ester in the presence of 30% methanol. A comparison of the activities on N-acetyl-L-lysine ethyl ester and N-benzoyl-L-lysine ethyl ester with and without methanol with data obtained by other investigators for chymotrypsinogens A, B, and C (22, 29) (Tables II and III) suggests that chymotrypsin 1 and 2 correspond with mammalian chymotrypsins A, B, and C (22, 29) (29).

**TABLE II**

| Substrate | Methanol concentration | Relative activity |
|-----------|------------------------|------------------|
| ATEE      | %                      | Chymotrypsin 1    |
| ATEE      | 30                     | 22               |
| BTEE      | 30                     | 21               |
| ATryEE    | 2.5                    | 21               |
| ATE       | 30                     | 21               |
| BTEE      | 30                     | 21               |
| ATryEE    | 2.5                    | 21               |
| BTEE      | 30                     | 21               |
| ATryEE    | 2.5                    | 21               |
| BTEE      | 30                     | 21               |

* Relative activity = (rate of hydrolysis of substrate/rate of hydrolysis of N-acetyl-L-lysine ethyl ester in buffer) × 100.

Activities on the various substrates were measured with the peak fractions of each chymotrypsinogen after activation with trypsin.

**TABLE III**

| Substrate | Relative activity |
|-----------|------------------|
| Bovine    | A A C 1 2       |
| Dog       | 1 A C 2         |
| Chicken   | 1 C 2           |

in all cases in the chymotrypsinogen 2 peak and most of the residual activity in the chymotrypsinogen 1 peak. Activities recovered from the chymotrypsinogen 3 peak varied considerably (from 0.2% to 10% of the total N-acetyl-L-lysine ethyl ester activity) from one zymogen granule extract to the next. Measurements of the activity of chymotrypsins 1 and 2 on different substrates in presence of varying concentrations of methanol revealed that the two peaks of chymotrypsinogen have different specificities (Table I). Chymotrypsin 2 is more active than chymotrypsin 1 on N-benzoyl-L-lysine ethyl ester, N-acetyl-L-lysine ethyl ester, and N-benzoyl-L-lysine ethyl ester. Moreover, the activity of chymotrypsin 1 on N-acetyl-L-lysine ethyl ester is greatly reduced in presence of 30% methanol, while chymotrypsin 2 retains much of its original activity. Both chymotrypsinogens 1 and 2 show very little activity on N-benzoyl-L-lysine ethyl ester in the presence of 30% methanol. A comparison of the activities on N-acetyl-L-lysine ethyl ester and N-benzoyl-L-lysine ethyl ester with and without methanol with data obtained by other investigators for chymotrypsins A, B, and C (22, 29) (Tables II and III) suggests that chymotrypsins 1 and 2 correspond with mammalian chymotrypsins B and A, respectively. Because of the small amounts of chymotrypsin 3 in most of the zymogen granule extracts studied, no accurate data are available on its specificity. However, preliminary studies indicate that it has high relative activity on N-benzoyl-L-lysine ethyl ester (about 60% of that on N-acetyl-L-lysine ethyl ester) and a low relative activity on N-acetyl-L-lysine ethyl ester (about 1% of its activity on

**TABLE I**

| Substrate | Methanol concentration | Relative activity |
|-----------|------------------------|------------------|
| ATEE      | %                      | Chymotrypsin 1    |
| ATEE      | 30                     | 22               |
| BTEE      | 30                     | 21               |
| ATryEE    | 2.5                    | 21               |
| ATE       | 30                     | 21               |
| BTEE      | 30                     | 21               |
| ATryEE    | 2.5                    | 21               |
| BTEE      | 30                     | 21               |

* Calculated from data of Folk and Schirmer (22).

* Relative activity = (activity on substrate/activity on N-benzoyl-L-lysine ethyl ester) × 100.

* Calculated from data in Table I.

# Relative activity = (rate of hydrolysis of substrate/rate of hydrolysis of N-acetyl-L-lysine ethyl ester in buffer) × 100.

Activities on the various substrates were measured with the peak fractions of each chymotrypsinogen after activation with trypsin.

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| Dog       | 1 A C 2         |
| Chicken   | 1 C 2           |
TABLE IV
Recoveries and degree of purification of enzymes and zymogens

| Enzyme | Units of enzyme Placed on column | Eluted from column | Recovery in zymogen granule extract | In column peak | Degree of purification |
|--------|---------------------------------|-------------------|-------------------------------------|----------------|-----------------------|
| Amylase | 39200                           | 38049             | %                                   | 97             | 392                   | 2350                   | 6.0               |
| Trypsinogen (p-toluene sulfonyl-L-arginine methyl ester) | | | | | | | |
| 1.     |                                  |                   |                                     | 24.8           | 5.7                   | 6.8                    |
| 2.     |                                  |                   |                                     | 51.0           | 40.0                  | 48.0                   |
| Total  |                                  |                   |                                     | 83.5           | 75.8                  | 91                     | 0.84              |
| (Pro)carboxypeptidase | 7800 | 7510 | 97 | 78 | 910 | 11.7 |
| Hippuryl-L-phenylalanine | 10.0 | 10.3 | 5.0 |
| Carboxypeptidase | 227.0 | 40.5 | 12.5 |
| Procarboxypeptidase | 43.5 | 7.7 | 2.4 |
| Total | 320 | 325.0 | 100 | 3.25 |
| Hippuryl-L-arginine (procarboxypeptidase B) | 58 | 50.5 | 88 | 0.58 | 26.8 | 46 |
| Chymotrypsinogen (N-acetyl-L-tyrosine ethyl ester) | 911 | 120 | 6.8 |
| 1.     |                                  |                   |                                     | 850            | 89                    | 5.0                    |
| 2.     |                                  |                   |                                     | 113            | 40                    | 2.2                    |
| Total  |                                  |                   |                                     | 1790           | 1874                  | 104                    | 17.9             |

* Procarboxypeptidase A peak.
* Degree of purification is a minimum value since it was calculated from over-all specific activity of all different forms of enzyme in the zymogen granule extract.
* Column peaks as shown in Fig. 1.

N-acetyl-L-tyrosine ethyl ester). The preferential specificity for N-benzoyl-L-leucine ethyl ester suggests that chymotrypsin 3 is a distinct molecular species from chymotrypsins 1 and 2 and that is may correspond with mammalian chymotrypsin C.

Other Enzymes—Amylase, which is probably the major protein component of chicken pancreas zymogen granules, is eluted from the column after all of the abovementioned proteolytic zymogens. Lipase was very unstable, rather low yields being obtained from the column. The major peak of lipase was usually eluted in the region of procarboxypeptidases B and A. Endonuclease activity was not eluted as a sharp peak but appeared in fractions from protein Peak 1 to protein Peak 5.

Yields and Degree of Purification of Enzymes and Zymogens—Yields of enzymes were uniformly high (Table IV). Purifications up to more than 40-fold relative to the zymogen granule extract were obtained. The specific activity of amylase in the peak fractions was about the same as that reported previously for the pure enzyme (5).

DISCUSSION
The data presented above give a picture of the spectrum of exportable proteins in the pancreas of an avian species and thus help to fill a gap in the knowledge of the comparative biochemistry of pancreatic hydrolases. Qualitatively, the composition of chicken pancreas zymogen granule contents is very similar to that of zymogen granule contents or pancreatic juices from other vertebrate species (1–4). The properties of amylase and of the proteolytic zymogens studied are compatible with the assumption that they are homologous with similar enzymes or zymogens in other species (cf. Reference 3). As has been pointed out previously, one chicken pancreas enzyme, namely the magnesium ribonuclease (endonuclease) is apparently not homologous with its bovine counterpart (ribonuclease) (8, 9, 12). Amylase from chicken pancreas is unusual in that it is strongly anionic whereas amylases from other species studied so far were either cationic or weakly anionic (1–4).

It is of interest that trypsin activity (on p-toluene sulfonyl-L-arginine methyl ester) in the chicken pancreas is only about 0.1 of that of chymotrypsin (on N-acetyl-L-tyrosine ethyl ester), whereas, in most species studied (1, 2, 4, 31–33) the relative amounts of trypsin and chymotrypsin are of the same order of magnitude. The presence of two trypsinogen peaks is not surprising as two or more trypsinogen peaks have been observed in preparations of zymogen granule contents from pancreas of rat (3), lungfish (4), salmon (31), and human beings (32, 33).

As in other species, procarboxypeptidases A and B were found which, after activation, preferentially hydrolyze peptide substrates with COOH-terminal aromatic and basic amino acid residues, respectively (1–4, 30, 36). There was little overlap in specificity towards peptide substrates between carboxypeptidases A and B (Fig. 2). Carboxypeptidase A had little or no activity on hippuryl-L-arginine or hippuryl-L-lysine, while carboxypeptidase B had little activity on hippuryl-L-phenylalanine or N-carbobenzoxyglycyl-L-phenylalanine. Thus, chick pancreas carboxypeptidase B apparently differs from the bovine and dogfish enzymes which showed considerable activity towards N-carbobenzoxyglycyl-L-phenylalanine (30, 36).

The data presented (Table I) show clearly that chymotrypsins 1 and 2 are distinct species of molecule which on activation give rise to chymotrypsins with different specificivities. The low activity of chymotrypsin 1 on N-acetyl-L-tryptophan ethyl ester in presence of 30% methanol (Tables I, II, and III) together with the low activity of both chymotrypsins 1 and 2 on N-benzoyl-L-leucine ethyl ester in presence of methanol (Tables I and II), indicate that chymotrypsins 1 and 2 correspond with chymotrypsins B and A, respectively (1, 18, 22, 29). The cationic chymotrypsinogen 1 is probably the zymogen corresponding to the cationic chicken chymotrypsin previously purified and studied by Ryan (6) and Ryan et al. (7). Our preliminary observations support the view that chymotrypsin 3 corresponds with a mammalian C-type chymotrypsin. This conclusion is based on its high relative activity on N-benzoyl-L-Leucine ethyl ester and low relative activity on N-acetyl-L-tryptophan ethyl ester.
ester (22). If this is indeed the case, then chick pancreas resembles pig and bovine pancreas in having A, B, and C-type chymotrypsins (22, 34). It may be significant that chymotrypsinogen 3 is eluted in the same region as the subsidiary procarboxypeptidase A, B, and C-type chymotrypsins (22, 34). It is possible, therefore, that in the present case chymotrypsinogen 3 and procarboxypeptidase occur in the form of a complex. Because of the small quantity of material available, it was not possible to determine whether chymotrypsinogen occurs as a complex with procarboxypeptidase or whether their similar elution patterns are a coincidence.

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