Activities of Chicken Pancreatic Proteinases Toward Synthetic Substrates.* (27637)

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Proteolytic enzymes of the bovine pancreas have been extensively studied, and their enzymic and physical properties are known in considerable detail. As Desnuelle(1) has recently pointed out, however, the investigation of similar enzymes in other animal species has been largely neglected. A few recent reports indicate the existence of some differences in the qualitative make-up and physical properties of bovine pancreatic proteinases and those from some other animal species(1-4), all but one of which were mammals(4). Although the chicken has been used extensively as an experimental animal in physiology and nutrition, proteolytic enzymes of the chicken pancreas have received little attention, and information concerning their activities toward synthetic substrates is lacking. This paper reports the behavior of pancreatic proteinases from the chicken toward several synthetic substrates.

Experimental. Materials. Synthetic substrates were purchased from Cyclo Chemical Corp., Nutritional Biochemicals Corp., Sigma Chemical Co., and Mann Research Laboratories. Dialyzed, lyophilized hemoglobin was obtained from Mann Research Laboratories and Worthington Biochemical Corp., and crystalline bovine trypsin from Armour Laboratories. Trypsin inhibitors were purchased from Nutritional Biochemicals Corp.

Enzyme sources. The source of chicken pancreatic proteinases consisted of either whole pancreatic homogenates or of fractions prepared therefrom by ammonium sulfate precipitation. Similar results were obtained with both types of enzyme preparation, but the data reported below were obtained using a preparation made as follows: Pancreases were removed from freshly slaughtered chickens, chilled, homogenized in cold deionized water, and strained through cheese cloth. The homogenate was made 0.2 saturated with ammonium sulfate, stored overnight at 5°C, then centrifuged at 22,000 × *g*. The precipitate was discarded, the supernatant solution was made 0.8 saturated with ammonium sulfate and allowed to stand at 5°C for 7 hours, then centrifuged at 22,000 × *g* for 20 minutes. The precipitate thus collected was dissolved in 0.001 M TRIS‡, pH 7.0, dialyzed in the cold against 0.001 M TRIS, lyophilized, and stored in a vacuum desiccator at −10°C. The desired weight of lyophilized chicken pancreas extract was dissolved in TRIS buffer for tryptic or chymotryptic assay, and in either TRIS or veronal for carboxypeptidase determinations.

To estimate nitrogen content of pancreatic preparations, a standard curve was made relating the color reaction of Lowry *et al.*(5) to nitrogen values obtained by micro-Kjeldahl analysis of a pancreatic homogenate. Nitrogen contents of all preparations were then estimated from this graph.

Enzyme assays. Proteolytic activity was determined by reaction with denatured hemoglobin(6), measured by absorbance at 280 mμ in a Beckman DU spectrophotometer. Trypsin-like activity was assayed with esters of *L*-arginine and *L*-lysine (Table I) by the potentiometric titration of Schwert *et al.*(7), in 0.002 M TRIS buffer which was 0.005 M in $CaCl_2$. It was also assayed with benzoyl-*L*-argininamide (BAA) by determining the ammonia liberated, using the quantitative ninhydrin procedure of Moore and Stein(8). Chymotrypsin-like activity was measured by the titrimetric procedure of Balls and Jansen (9), using as substrates a number of esters of *L*-tyrosine and *L*-phenylalanine (Table I). Carboxypeptidase-like activity was measured

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‡ TRIS—(hydroxymethyl) aminomethane.

TABLE I. Activities of Chicken Pancreatic Enzymes toward Synthetic Substrates for Proteolytic Enzymes.*

Compound†	Activity (μmoles hydrolyzed/min/mg N)
Trypsin substrates:	
TSAME	18.5
BAEE	2.8
BAA	.33
LME	1.0
Chymotrypsin substrates:	
ATEE	220.
APEE	89.2
TEE	31.1
PEE	10.4
Carboxypeptidase substrates:	
CGP	.2

* These activities are to be regarded as illustrative, since rates vary with the activation procedure, the individual pancreatic preparation, and assay conditions. All tests reported in the above table were made on the same pancreatic preparation.

† The following abbreviations are used: TSAME = *p*-toluenesulfonyl-*L*-arginine methyl ester; BAEE = N-benzoyl-*L*-arginine ethyl ester; BAA = N-benzoyl-*L*-arginine amide; LME = *L*-lysine methyl ester; ATEE = N-acetyl-*L*-tyrosine ethyl ester; APEE = N-acetyl-*L*-phenylalanine ethyl ester; TEE = *L*-tyrosine ethyl ester; PEE = *L*-phenylalanine ethyl ester; and CGP = N-carbobenzoxyglycyl-*L*-phenylalanine.

by the quantitative ninhydrin procedure, with a substrate consisting of 0.02 M carbobenzoxyglycyl-*L*-phenylalanine (CGP) in 0.02 M veronal buffer, pH 7.5.

Results. As shown in Table I and Figs. 1 and 2, the pancreatic preparations actively hydrolyzed denatured hemoglobin and a number of compounds known to be substrates for bovine trypsin, chymotrypsin, and carboxypeptidase. Hydrolysis of hemoglobin and synthetic substrates, however, was dependent upon activation of zymogens, which was accomplished by incubating the enzyme source with crystalline bovine trypsin. Autolysis of homogenates or pancreas fractions was a relatively poor method for activation. In trypsin assays, activity of the added bovine trypsin was determined separately and subtracted from the total activity.§ In a number of experiments, the ratio of added bovine trypsin

§ The proportion of total activity contributed by the added bovine trypsin varied with the amount added, of course, and with the conditions used in activation. Although the fraction of total activity attributable to the enzymes in chicken pancreas varied somewhat between experiments, it was always detectable and easily measured.

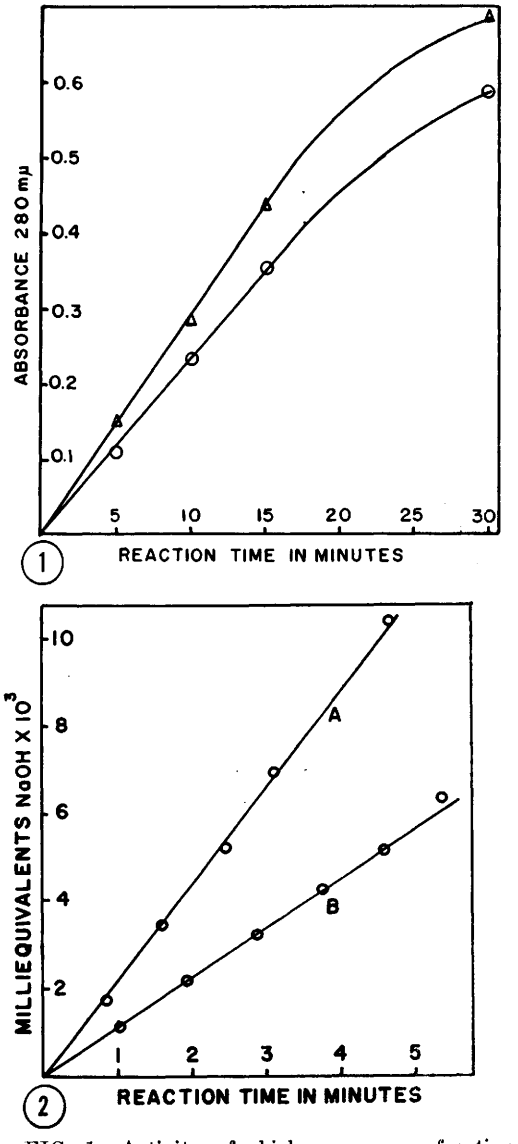

FIG. 1. Activity of chicken pancreas fraction toward denatured hemoglobin, showing effects of activation. △ = 8.66 hr activation; ○ = 2.5 hr activation. One μg crystalline bovine trypsin per 45 μg of pancreas preparation used for activation; 1 ml of activated pancreas preparation plus 5 ml hemoglobin substrate used for enzyme activity tests. Values shown have been corrected for the trypsin added for activation. No proteolysis occurred without activation.

FIG. 2. Hydrolysis of esters by activated chicken pancreas preparation. A = *L*-tyrosine ethyl ester; B = *p*-toluenesulfonyl-*L*-arginine methyl ester. In B, correction has been made for the bovine trypsin used in activation.

to chicken pancreatic preparations was varied, as was the time allowed for activation, but no attempt was made to define rigorously all of the conditions for optimum activation. As an example, however, incubating 1 μg of crystalline trypsin per 50 μg of pancreatic extract at 25°C gave maximum activity toward a chymotrypsin substrate (TEE) after approximately 2 hours, while greatest activation of the pancreatic preparation toward a trypsin substrate (TSAME) occurred at slightly more than 8 hours. The shape of the activation curve for chymotryptic activity suggested the formation of a highly active transitory form, possibly comparable to bovine π-chymotrypsin, which was rapidly converted to a slightly less active form, perhaps analogous to bovine α-chymotrypsin.

The relative activities toward various trypsin and chymotrypsin substrates are in general similar to those shown by bovine enzymes (10), and in our preparations, the total activity toward chymotrypsin substrates was greater than that toward trypsin substrates, as it is in bovine pancreatic juice(11). Because of the heterogeneity of the enzyme preparation, no attempt was made to draw kinetic inferences from the data. The chicken pancreas preparation hydrolyzed TSAME at an optimum rate between pH 8.5 and 9.0; the optimum pH range for ATEE activity was from 8.2 to 8.7.

Incubation of the enzyme preparation in 10^{-3} M diisopropylphosphofluoridate for 40 to 55 minutes at room temperature completely destroyed activity toward both TSAME and TEE. These results indicate a similarity between the active centers of the chicken enzymes and bovine trypsin and chymotrypsin. The effects of 3 naturally occurring inhibitors of bovine trypsin were also tested by incubating them with the chicken pancreatic enzyme preparation for 3 minutes before addition of the substrate. This treatment greatly reduced activity toward both TSAME and TEE (Table II). The inhibition of TEE activity appears unexpectedly large, if one assumes that the chicken enzymes behave in a manner similar to bovine trypsin and chymotrypsin. However, Croston(4) reported a similar effect with soybean trypsin inhibitor on the mixed pancreatic enzymes of the chinook salmon, and offered evidence that the trypsin-inhibitor complex was responsible for reduction of the activity towards a chymotrypsin substrate.

TABLE II. Effects of Naturally-Occurring Inhibitors of Bovine Trypsin on Esterolytic Activities of Chicken Pancreatic Enzymes.

Trypsin inhibitor	Amount (μg/mg of enzyme preparation)	% inhibition of activity toward: TSAME	TEE
Soybean	75.0	77	45
Pancreatic	37.5	73	24
Lima bean	75.0	84	79

Discussion. The esterase and amidase activities of chicken pancreatic proteinases appear to be similar to those of the bovine enzymes, as indicated by their behavior toward synthetic substrates and by the effects of inhibitors. It has been more or less tacitly assumed that the pancreatic juices of all animal species contain zymogens which give rise to enzymes identical to bovine trypsin, chymotrypsin and carboxypeptidase(1). The limited information available indicates a similarity between the catalytic specificities of the pancreatic enzymes from different species. However, extensive investigations of bond specificities from different species have not been made, and differences may be revealed with further work. On the other hand, species differences exist in the qualitative makeup of pancreatic proteinases. Marchis-Mouren *et al.*(2) have reported that the pancreatic juices of the pig and dog contain only one chymotrypsinogen, in contrast to bovine pancreatic juice, which contains two. Buck *et al.*(3) reported differences in the stability of trypsins from swine, cattle, and sheep, and Croston(4) found the tryptic enzymes of the chinook salmon to be inactivated at the low pH values commonly used in isolating bovine trypsin and chymotrypsin. These data suggest the existence of structural differences in proteolytic enzymes of various species, and indicate the desirability of further comparative biochemical studies with the enzymes of different species.

Summary. A study was made of the activity of chicken pancreatic enzymes toward

synthetic substrates known to be hydrolyzed by bovine trypsin, chymotrypsin, and carboxypeptidase. When activated with bovine trypsin, preparations from chicken pancreas hydrolyzed all of the synthetic substrates tested, and also exhibited proteolytic activity toward denatured hemoglobin. Unactivated preparations exhibited little or no activity toward proteins or synthetic substrates. Trypsin-like and chymotrypsin-like activities were inhibited by diisopropylphosphofluoridate, and by naturally occurring trypsin inhibitors from soybeans, lima beans, and pancreas. The optimum activity for hydrolysis of trypsin substrates was at pH 8.5 to 9.0, and for chymotrypsin substrates was from pH 8.3 to 8.7. The significance of comparative biochemical studies of proteolytic enzymes of different species is discussed.

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Specificity of an Intestinal Lipase for Monoglycerides.* (27638)

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In a recent study in this laboratory an enzyme was observed in rat intestinal mucosa which appeared specific for the hydrolysis of monoglycerides(1). Lipases in the succus entericus have been previously demonstrated to resemble the lipase of the pancreas in that they were active as hydrolytic enzymes for triglycerides(2). Nikkila *et al.*(3) demonstrated the clearing of olive oil emulsions with a saline extract of rat duodenum, and Schmidt *et al.*(4) described an enzyme in extracts of rat intestine which could promote the splitting of monoglycerides. More recently DiNella *et al.*(5) have demonstrated a lipase from hog duodenum which hydrolyzed tri-, di-, and monoglycerides at approximately equal rates. Hübscher and Clark(6) and Senior and Isselbacher(7) have observed hydrolysis of monoglycerides by rat intestinal mucosal fractions in studying the pathway of triglyceride synthesis. While there is ample evidence for the occurrence of a lipase in the small intestine, its exact role in fat digestion is not clear.

In this study an enzyme from the homogenized mucosa of the rat small intestine has been shown to hydrolyze monoglycerides of long chain saturated and unsaturated fatty

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