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Studies on the Proteolytic Enzyme System of the Pancreas

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Introduction

The existence of an enzyme in pig pancreas designated as protaminase was described years ago by Waldschmidt-Leitz *et al.* (1) and by Weil (2). The activity of this enzyme appeared to be confined to the hydrolysis of free arginine from the C-terminal end of protamines. In view of the great advances made in the field of proteolytic enzymes since the publication of the above work, it was highly desirable to reinvestigate this problem in the light of recent findings. Evidence presented in this paper demonstrates that protaminase is not identical to the known proteolytic enzymes of the pancreas. Additional findings would also indicate the existence of a second new proteolytic enzyme.

Experimental and Results

Preparation of Enzyme Stock Solution

Pancreas glands of newly killed pigs were immediately ground and placed in a tenfold amount of acetone. After standing at room temperature 1 hr., the suspension was filtered by suction, the gland residue was resuspended in the same solvent, and this operation was repeated three times. The same procedure was then repeated three times with acetone–ether (1:1) and finally three times with ether alone. The final gland residue, dehydrated and defatted in this manner, was dried at room temperature and passed through a fine screen to remove the connective tissue. The resulting gland powder was then suspended in 80% glycerol (1:10), and, after standing 1 day at room temperature, was filtered by gravity through a fluted filter (Whatman No. 14). The glycerol filtrate obtained in this fashion was used as the stock enzyme solution and was stored at 4°. It was found to be highly stable, and no changes could be observed in its various enzymic activities over a period of a year.

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[1] A laboratory of the Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

To separate the various enzymes of the glycerol–pancreas extract, the adsorption procedure of Waldschmidt-Leitz *et al.* (1) was employed, using a C_γ-aluminum hydroxide suspension prepared according to Willstätter and Kraut (3). It contained 26 mg. of dry residue/ml. after drying at 110°.

A typical example of the separation procedure is given: To 50 ml. of glycerol extract were added 50 ml. of water and 7 ml. of a 1 *M* acetate buffer of pH 3.8. This solution was adsorbed five times batchwise with 5 ml. of C_γ suspension under ice cooling and centrifuged each time in a refrigerated centrifuge at 4°. The adsorption at acid pH completely removed the aminopeptidase activities. The supernatant enzyme solution was subsequently adjusted to pH 7.0 with 1 *N* NaOH and adsorbed eight times with 7 ml. of the same adsorbent. This latter step adsorbed completely the carboxypeptidase activity. The total volume after adsorption amounted to 124 ml.

In order to follow the proteolytic enzyme distribution during the adsorption procedure, the following enzymic assays were employed (in all the assays aliquots containing 0.4 ml. of the original glycerol extract were used).

Aminopeptidase

To aliquots of enzyme solution, 1 ml. of 0.2 *M* phosphate buffer of pH 8.0 and 2 ml. of a 0.05 *M* L-leucylglycine (L-G) or L-leucyldiglycine (L-G-G) solution were added, made up to 4 ml.; after incubation at 30° for the given time, the increase in α-NH_2N groups was measured by the Van Slyke procedure.

Carboxypeptidase

To the enzyme solution 1 ml. of a 0.2 *M* phosphate buffer of pH 8.0 and 2 ml. of 0.05 *M* solution of chloroacetyl-L-tyrosine (Cl-Ac-T) were added and made up to a volume of 4 ml. After the given time of incubation at 30° the increase in α-amino groups was measured as above.

Chymotrypsin

The assay was carried out according to Schwert *et al.* (4) using 10 ml. of a 0.025 *M* solution of *N*-acetyl-L-tyrosine ethyl ester (AcTEE) at 25°.

Trypsin

The method employed was the same as for chymotrypsin except that *p*-toluene sulfonyl-L-arginine ethyl ester (TSAEE) was used as substrate.

Protaminase

To 2 ml. of salmine sulfate solution containing 40 mg. and adjusted to pH 8.0, 1 ml. of 0.2 *M* phosphate buffer of pH 8.0 and the enzyme solution were added and made up to a volume of 4 ml. After incubation at 30°, the increase in α-amino groups was measured as described before.

Toluene was added to all reaction mixtures.

TABLE I

Distribution of Proteolytic Enzymes in Glycerol Extracts of Fresh Pancreas Glands after Successive Purification Steps

Enzyme tested	Substrate used	Incubation time	Trypsin activation[a]	Activity (ml. of 0.1 *N* NaOH) after successive purification steps			
				1. Original extract	2. After adsorption at pH 3.8	3. After adsorption at pH 7.0	4. After DFP-inhibition
		min.					
Dipeptidase	L-G	60	N	0.70	0.00	0.00[b]	0.00[b]
			T	0.70	0.00	0.00[b]	0.00[b]
Polypeptidase	L-G-G	60	N	0.88	0.00	0.00[b]	0.00[b]
			T	0.90	0.00	0.00[b]	0.00[b]
Carboxypeptidase	Cl-Ac-T	120	N	0.36	0.23	0.00[b]	0.00[b]
			T	0.72	0.58	0.00[b]	0.00[b]
Trypsin	TSAEE	60	N	0.00	0.00	0.00	0.00[c]
			T	0.00	0.00	0.00	0.00[c]
Chymotrypsin	AcTEE	5	N	0.22	0.08	0.02	0.00[c]
			T	2.04	1.82	1.10	0.00[c]
Protamine-hydrolyzing enzyme	Salmine sulfate	360	N	—	—	0.28	0.30
			T	—	—	0.85	0.87

[a] N = not activated; T = activated with trypsin.
[b] Tested after 24 hr. of incubation at 30°.
[c] Tested after 2 hr. of incubation at 25°.

Distribution of the Proteolytic Enzymes of the Pancreas–Glycerol Extract during Adsorption

The distribution of the pancreatic proteolytic enzymes during the several steps of separation is presented in Table I. Since the glycerol extract was derived from fresh pancreas glands, carboxypeptidase, chymotrypsin, and trypsin should be present chiefly in their inactive form; therefore, Table I also includes these activities after trypsin activation.

Typical examples of activation are given: To 10 ml. of enzyme solution was added 5 ml. of 0.2 *M* phosphate buffer of pH 8.0 and 0.1 ml. of a trypsin solution containing 0.025 mg., and this solution was incubated for 24 hr. at 4°. For inhibition with isopropyl phosphorofluoridate [diisopropyl fluophosphate (DFP)], the conditions were the same as above except that 0.1 ml. of a 0.05 *M* DFP solution was added either before or after trypsin activation and incubated for 24 hr. at 4°.

Results presented in Table I show that the original glycerol extract

contains all the known pancreatic proteolytic enzymes except trypsin. A considerable increase in carboxypeptidase and chymotrypsin activity was observed after activation by traces of trypsin, while at the same time no tryptic activity could be detected. The complete absence of trypsin and trypsinogen from the glycerol extract might be due to either their destruction during extract preparation or their insolubility in glycerol. Adsorption with C_γ at pH 3.8 removes the dipeptidase and polypeptidase activities with only moderate changes in the other activities. Successive adsorption with C_γ at pH 7.0 brings about the complete removal of carboxypeptidase and a partial decrease in chymotrypsin activities.

As Table I shows, at this point the glycerol extract purified by the above procedure was free of peptidase, carboxypeptidase, and trypsin activities but still possessed chymotrypsin activity and the ability to hydrolyze salmine sulfate. Addition of DFP to this solution as described before resulted in complete inhibition of the remaining chymotrypsin activity but essentially no change in the salmine-hydrolyzing ability of this preparation. The enzyme solution prepared in this manner will be referred to in this paper as protaminase.

pH Optimum of Protaminase

A protaminase solution described for Table I was used. To 1-ml. aliquots of protaminase adjusted to pH's 5.0, 6.0, 7.0, 8.0, and 9.0 were added 1 ml. of 0.2 *M* citrate-phosphate buffer of the corresponding pH and 2-ml. aliquots of a salmine sulfate solution containing 40 mg. which was adjusted similarly to the desired pH. After 7 hr. incubation at 30° the increase of α-amino groups was measured by the Van Slyke method. The results obtained and presented in Fig. 1 show a pH optimum of about 8.0.

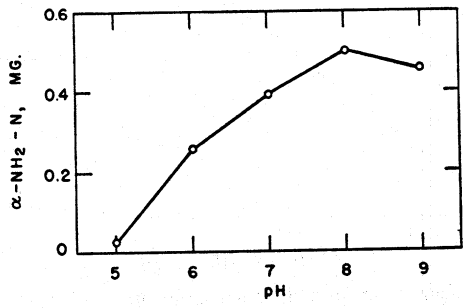

FIG. 1. pH optimum of protaminase.

It has been reported previously (1) that protaminase exerts its action on protamines by splitting off the C-terminal arginine from the protein molecule. The method used was based on the extraction of free arginine with 80% methyl alcohol. In investigating this problem, the manometric Warburg method (5, 6) was employed for determining the free arginine liberated during the hydrolysis of salmine and clupein sulfate by protaminase.

The reliability of this method for the present purpose was tested as follows: To 1 ml. of L-arginine.HCl solution adjusted to pH 8.0, containing increasing amounts of this amino acid, was added 1 ml. purified beef arginase solution (containing 25 units) which was then incubated for 40 min. at 30°. Following this, the reaction mixture was placed in boiling water for 5 min. After cooling, a 1-ml. aliquot was transferred to a Warburg vessel; to this was added 1 ml. of 3 *M* acetate buffer of pH 5.0 and, in the side arm, 0.5 ml. of urease solution dissolved in 1.5 *M* acetate buffer of pH 5.0. (The urease stock solution was freshly prepared by suspending 400 mg. of Jack Bean urease in 10 ml. of 1.5 *M* acetate buffer of pH 5.0 and filtering after standing 2 hr. at room temperature.) After temperature equilibrium at 30° was attained, the contents of the vessel were mixed and the CO_2 evolution measured manometrically. Identical experiments were carried out in the presence of 40 mg. of salmine sulfate. The results obtained and presented in Fig. 2

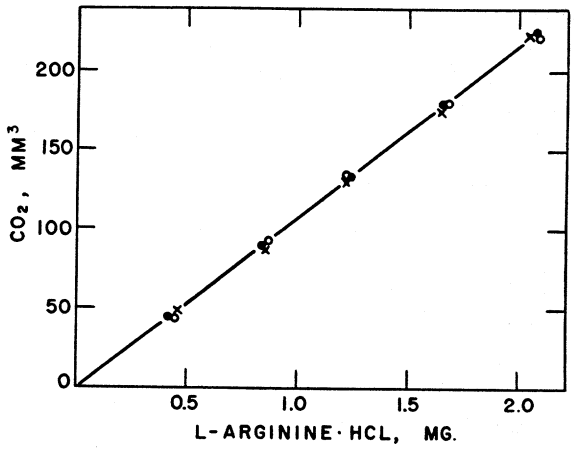

FIG. 2. Manometric determination of free arginine. ●——● theoretical value; ○——○ arginine; ×——× arginine and salmine sulfate.

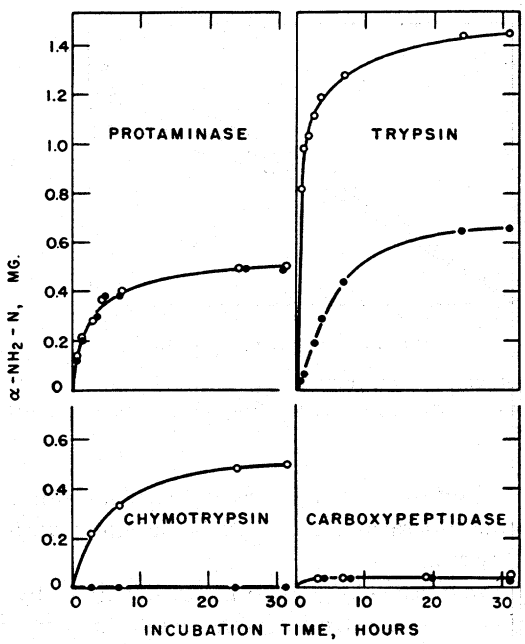

FIG. 3. Hydrolysis of salmine sulfate with various enzymes. ○——○ total α-NH_2N liberated; ●——● free arginine-α-NH_2N liberated.

show that complete recovery of arginine was achieved even in the presence of salmine sulfate. After thus establishing the reliability of the above procedure, the action of protaminase on salmine and clupein sulfate in the production of free arginine was investigated. In order to compare the mode of action of protaminase with that of trypsin, chymotrypsin, and carboxypeptidase, the same experiments were carried out with these enzymes.

Enzyme preparations used in experiments presented in Fig. 3 were as follows: Protaminase was prepared as described for Table I and Fig. 1; for the rest of the experiments crystalline chymotrypsin, carboxypeptidase (treated with DFP), and trypsin were used. The digestion mixture was set up in such a manner that the 2-ml. aliquots used for Van Slyke determination contained 1 mg. of the crystalline enzyme (or 1 ml. of protaminase) and 40 mg. of salmine sulfate, and the total phosphate buffer (pH 8.0) concentration was 0.05 M. The method described above was used to determine the free arginine liberated during the digestion at 30° (Fig. 2). Results obtained and presented in Fig. 3 show that the

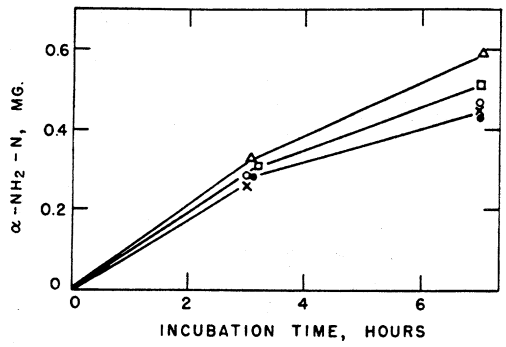

FIG. 4. Hydrolysis of various protamines by protaminase. ○——○ salmine sulfate; ●——● clupein sulfate; △——△ irridine. HCl; ×——× truttine.HCl; □——□ fontinine.HCl.

total α-NH_2 N liberated by the action of protaminase on salmine sulfate was due to free arginine liberation. The same experiment with clupein sulfate gave identical results.

In contrast to protaminase, chymotrypsin, although it hydrolyzed salmine sulfate, did not form any free arginine, while carboxypeptidase hardly acted on this substrate. Trypsin hydrolyzed salmine sulfate with a very high velocity accompanied by liberation of free arginine, the latter of which was produced chiefly after extensive hydrolysis. This observation with trypsin is in contradiction to earlier findings of Portis and Altman (7) who did not observe any free arginine formation.

Action of Protaminase on Various Proteins

The protaminase solution was prepared as described in experiments presented in Table I. The action of this enzyme was tested on various protamines in addition to salmine sulfate, such as clupein sulfate, irridine.HCl,[2] truttine.HCl,[2] and fontinine.HCl,[2] and the results obtained are presented in Fig. 4. Findings achieved with α-lactalbumin,[3] β-lactoglobulin,[3] casein, and gelatin are given in Fig. 5*A*. To determine the increase in α-NH_2 N, 4-ml. aliquots containing 40 mg. of protein, 1 ml. of protaminase, and 1 ml. of 0.2 *M* phosphate buffer of pH 8.0 were used. Results presented in Fig. 4 show that all protamines tested were hydrolyzed by protaminase in contrast to the findings presented in Fig. 5*A*

[2] Obtained through the courtesy of Dr. Kurt Felix, University of Frankfurt, Germany.

[3] Kindly supplied by Dr. W. G. Gordon of this laboratory.

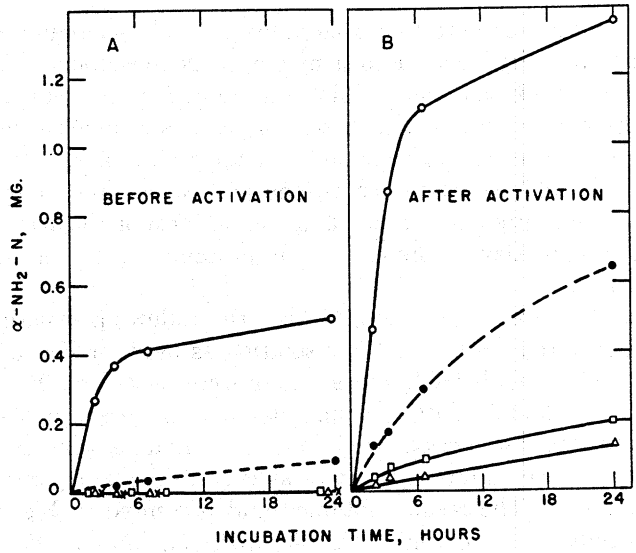

FIG. 5. *A*. Hydrolysis of various proteins by protaminase before tryptic activation. *B*. Hydrolysis of various proteins after tryptic activation. ○——○ salmine sulfate; ●——● casein; △——△ α-lactalbumin; ×——× β-lactoglobulin; □——□ gelatin.

in which α-lactalbumin, β-lactoglobulin, and gelatin were not hydrolyzed at all, while casein was cleaved to only a slight degree.

Activation of Protaminase Preparation by Traces of Trypsin

Since the glycerol extract used was prepared from fresh glands, the question arose as to whether protaminase might be partially present in the form of its inactive precursor. To answer this question the activity of protaminase was compared before and after activation by traces of trypsin. Protaminase was prepared as described for Table I using the solution resulting after C_γ adsorption at pH 7.0. To 50 ml. of enzyme solution, 25 ml. of 0.2 *M* phosphate buffer of pH 8.0 and 0.25 mg. of crystalline trypsin was added. For complete activation this solution was left overnight at 4°. To this solution then was added 0.5 ml. of 0.05 *M* DFP solution and left again overnight at 4°. For comparison an identical enzyme solution was prepared but without the addition of trypsin. Both enzyme solutions were free of trypsin, chymotrypsin, and carboxypeptidase activities. The action of the enzyme on various proteins was tested in the manner described for Fig. 4. Results obtained are presented in Fig. 5*A* and 5*B*.

It is of interest to note that α-lactalbumin and β-lactoglobulin which were inert, were hydrolyzed to a moderate degree upon activation of the enzyme. In like manner salmine sulfate and casein which were hydrolyzed to varying degrees, underwent an extensive increase in hydrolysis upon activation of the enzyme. The activation of the protaminase preparation could be attributed to one of two possible factors: either protaminase was present in a partially active form or the activation was due to a second enzyme present in the protaminase preparation in its precursor form.

To decide between these possibilities, the following experiment was carried out: Activated protaminase solution as used for Fig. 5*B* was adjusted to pH 2.3 with HCl, and aliquots were heated at 95° for 0, 10, 20, 30, 40, and 60 min.; after cooling at room temperature, the pH was adjusted to 8.0. The action of the enzyme samples thus treated was tested for salmine sulfate hydrolysis as described for experiments presented in Fig. 4. The results obtained and presented in Fig. 6 show a marked decrease in salmine sulfate-hydrolyzing ability after 10 min.

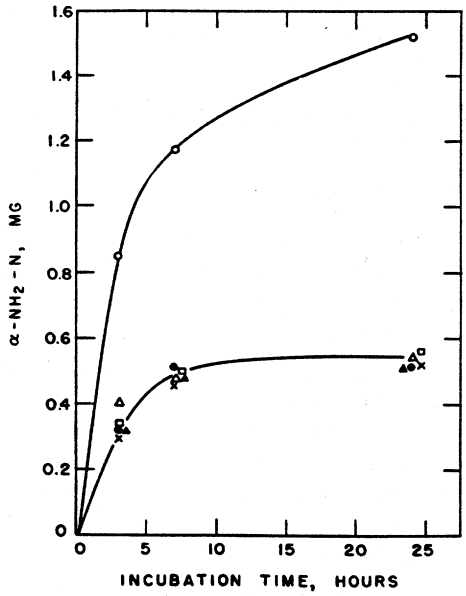

FIG. 6. Effect of heating period at 95° at pH 2.3 on the activity of the activated protaminase on salmine sulfate. ○——○ 0 min.; ●——● 10 min.; ×——× 20 min.; □——□ 30 min.; ▲——▲ 40 min.; △——△ 60 min.

heating at 95° and with no further changes even if the heating period was extended to 60 min. The pH optimum of this residual activity measured on salmine sulfate as substrate and using the conditions described for Fig. 1 was found to be about 8.0.

The above findings would exclude the first possibility and support the contention that activated protaminase might contain a second, heat-resistant enzyme, present originally in its inactive form.

To decide whether this heat-resistant residual activity of the protaminase preparation was due to protaminase (described in experiments of Fig. 3) or to a new activity due to the tryptic activation, the heat-resistant enzyme's ability to hydrolyze salmine sulfate and liberate free arginine from this substrate was compared to that obtained before heat treatment. The experimental procedure was the same as described for findings given in Fig. 3. The results obtained and presented in Fig. 7 show that while the activated protaminase preparation produces an extensive hydrolysis of salmine sulfate with a marked formation of free arginine (the latter of which is equivalent to the total α-NH_2 N increase at the early stages of the hydrolysis), the heat-resistant residual activity, although it hydrolyzed this substrate, did not result in any free arginine

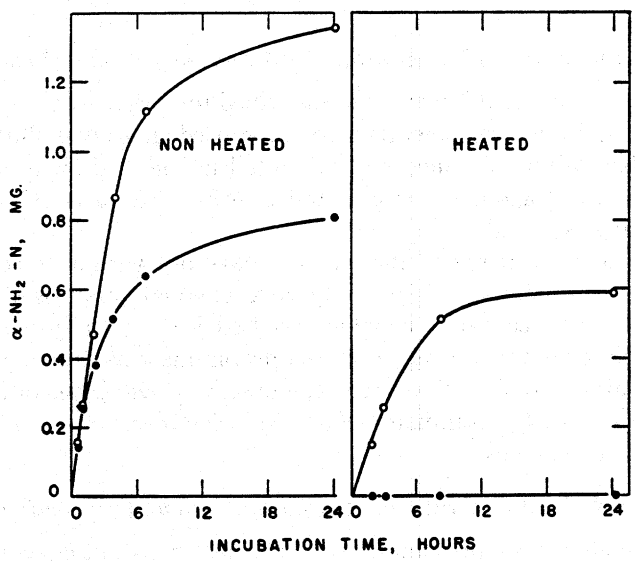

FIG. 7. Hydrolysis of salmine sulfate by nonheated and heated activated protaminase. ○——○ total α-NH_2N; ●——● free arginine-α-NH_2N.

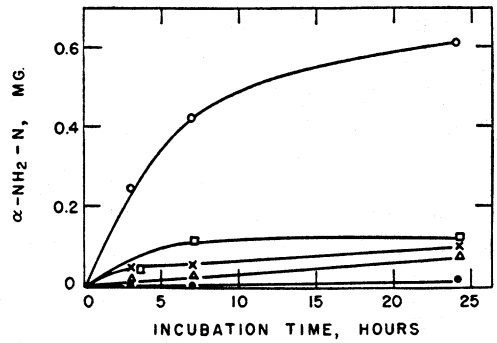

FIG. 8. Hydrolysis of various proteins by the "heat-resistant" enzyme. ○——○ salmine sulfate; ●——● α-lactalbumin; ×——× β-lactoglobulin; △——△ casein; □——□ gelatin.

formation. The results presented in Fig. 7 would indicate that the heat treatment brought about the destruction of protaminase (Fig. 3) and left behind an activity which might be due to a second enzyme. In support of the above conclusion, one should mention the fact that the protaminase solution heated under the same conditions underwent total destruction.

Action of the "Heat-Resistant" Enzyme on Various Proteins

The "heat-resistant" enzyme as described for Fig. 7 was used. Experimental conditions were identical to those used in studies illustrated in Fig. 5. The results obtained and presented in Fig. 8 show a very slight action on β-lactoglobulin, casein, and gelatin, while α-lactalbumin was not hydrolyzed at all.

Identical experiments carried out as above but with a "reconstituted activated protaminase," prepared by mixing equal amounts of protaminase and "heat-resistant" enzyme, resulted in essentially identical results as those obtained using activated protaminase as shown in Fig. 5*B*. These findings would indicate that the observed hydrolysis of these proteins by activated protaminase (Fig. 5*B*) is the result of the joint action of these two enzymes.

Stepwise Hydrolysis of Salmine Sulfate by Various Proteolytic Enzymes

The nonidentity of protaminase and the heat-resistant enzyme (Fig. 7) with trypsin or chymotrypsin was further strengthened by the following experiments: salmine sulfate was hydrolyzed exhaustively with trypsin

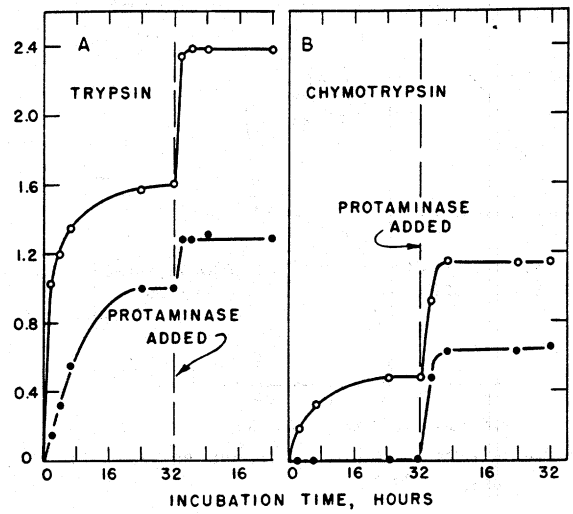

FIG. 9. Stepwise hydrolysis of salmine sulfate by various enzymes. ○——○ total α-NH_2N; ●——● free arginine-α-NH_2N.

and chymotrypsin, respectively, and, after 10 min. heat inactivation at 95°, a solution of protaminase prepared as described in experiments presented in Fig. 3 was added. The experimental conditions for determining total α-NH_2 N and free arginine were those as given for Fig. 3. Results obtained and shown in Fig. 9 indicate that after exhaustive hydrolysis of salmine by trypsin the addition of protaminase produced a definite increase in both total α-NH_2 N and free arginine, although the former was in excess of the free arginine formed (Fig. 9*A*). Exhaustive hydrolysis of salmine sulfate by chymotrypsin (as shown before in Fig. 3) produced no free arginine; addition of protaminase, nevertheless, resulted in an additional increase in total α-amino groups which was due entirely to free arginine formation (Fig. 9*B*).

Similarly, experiments were carried out in which salmine sulfate was hydrolyzed first with protaminase and then with the heat-resistant enzyme (described in experiments presented in Fig. 7), and a second experiment was performed in which the succession of enzymes was reversed. The results obtained are demonstrated in Fig. 10 and show that in the first case (Fig. 10*A*) the hydrolysis produced by protaminase was due entirely to free arginine formation, while the addition of the heat-resistant enzyme, although it further hydrolyzed the substrate, did not

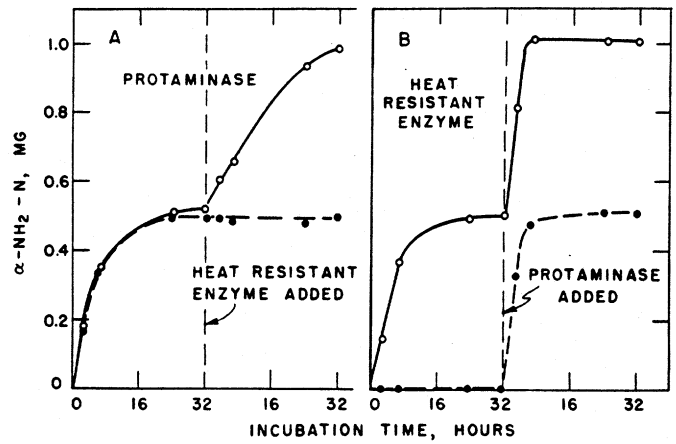

FIG. 10. Stepwise hydrolysis of salmine sulfate by various enzymes. ○——○ total α-NH_2N; ●——● free arginine-α-NH_2N.

result in any additional free arginine formation. The reversal of enzyme addition reversed the above findings (see Figs. 3 and 7).

Additional experiments were carried out in which salmine sulfate was hydrolyzed first by trypsin and chymotrypsin, respectively, and then by the heat-resistant enzyme (Fig. 7). The results obtained and presented in Fig. 11 show that addition of the heat-resistant enzyme to the tryptic digest of salmine sulfate produced only a slight increase in total α-NH_2 N but with no change in free arginine. Addition of this enzyme to the chymotrypsin digest of salmine sulfate, however, resulted in a

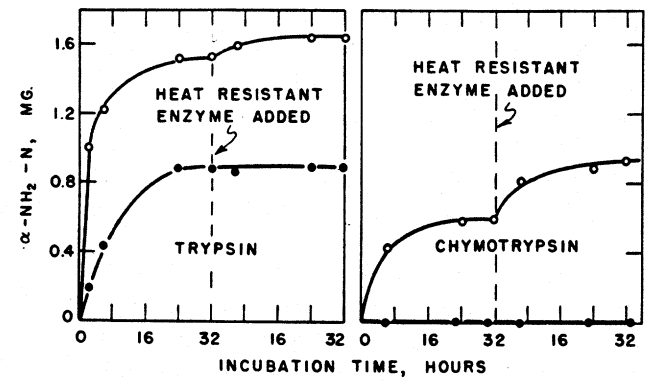

FIG. 11. Stepwise hydrolysis of salmine sulfate by various enzymes. ○——○ total α-NH_2N; ●——● free arginine-α-NH_2N.

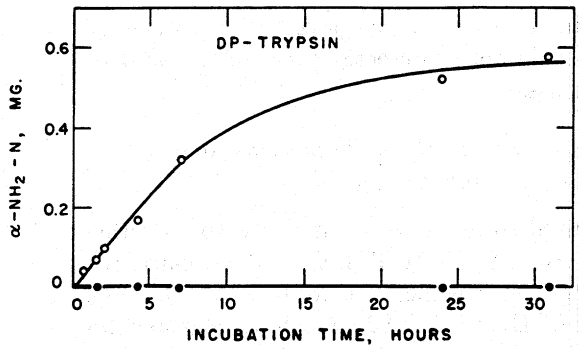

FIG. 12. Hydrolysis of salmine sulfate by DP-trypsin. O——O total α-NH_2N; ●——● free arginine-α-NH_2N.

distinct increase in total α-NH_2 N, while no free arginine formation could be observed.

Studies on the Possible Identity of the Heat-Resistant Enzyme with DFP-Inhibited Trypsin

In our preliminary note (8) it was reported that DFP-inhibited trypsin still exerted hydrolytic action on salmine sulfate, in contrast to chymotrypsin which was completely inhibited under these conditions. This residual activity of DP-trypsin was ascribed at the time to a possible second active site on the enzyme which was not affected by the inhibitor. A typical example of the action of DP-trypsin on salmine sulfate is presented in Fig. 12. Experimental conditions were those described for studies presented in Fig. 3. Comparison of salmine sulfate hydrolysis by DP-trypsin (Fig. 12) with that by trypsin (Fig. 3) shows that, while the latter produced a rapid and extensive hydrolysis of salmine sulfate accompanied by considerable free arginine formation, DP-trypsin resulted in a marked but greatly reduced hydrolysis of this protein, without any free arginine formation.

Subsequent experiments have shown that the observed hydrolysis of salmine by DP-trypsin was due to minute traces of uninhibited trypsin present in about 0.1 % concentration under the conditions of the experiment. In view of the above findings, therefore, we wish to correct the conclusions drawn in our preliminary note (8).

Since the hydrolysis of salmine sulfate by DP-trypsin (Fig. 12) and by the heat-resistant enzyme (Fig. 7) brought about essentially the same results, the possibility was considered that the latter might also be due

to traces of uninhibited trypsin. This possibility, nevertheless, was eliminated, since trypsin activity could not be detected even under the most rigorous conditions of esterase assay.

Preliminary Studies on the Specificity of Protaminase and the Heat-Resistant Enzyme

The following substrates tested so far were not hydrolyzed by either of the two enzymes: TSAEE, AcTEE, Cl-Ac-T, L-G, L-G-G, L-arginyl-L-leucine,[4] carbobenzoxy-L-arginyl-L-leucine,[4] histidyl-L-histidine, and α-lactalbumin. Except for TSAEE and AcTEE which were tested for 2 hr., the other substrates were reacted for 24 hr. at 30°.

In addition to the above substrates, protaminase did not hydrolyze β-lactoglobulin and gelatin (Fig. 5*A*) while the heat-resistant enzyme produced a very slight breakdown of these substrates (Fig. 8). A very slight hydrolysis was observed with both enzymes in the case of casein and poly-L-lysine[5] ($n = 38$).

Both enzymes hydrolyzed salmine and clupein sulfates greatly. In addition to the above protamines, protaminase also hydrolyzed irridine, truttine, and fontinine (Fig. 4).

Discussion

Evidence is presented that the pancreas protaminase activity described years ago (1, 2) is not associated with any of the known enzymes of the pancreas. Quantitative studies have shown that the action range of this enzyme is confined to the liberation of free arginine from the salmine and clupein molecules. Since it was established that these proteins possess an N-terminal proline and a C-terminal arginine (1, 9–14), it was concluded that the observed free arginine was liberated from the C-terminal end of the molecule in agreement with similar earlier observations (1, 2).

Protaminase described in this paper resembles the recently described "basic carboxypeptidase" of Folk (15); further investigations, however, are necessary to decide this possibility.

The action of protaminase is not confined to the intact salmine molecule but also can extend to the tryptic and chymotryptic breakdown

[4] Kindly supplied by Dr. C. W. Anderson of the American Cyanamid Co., Stamford, Conn.

[5] Kindly supplied by Kremers-Urban Co., Milwaukee, Wisconsin.

products. In both cases the action of protaminase was accompanied by the liberation of free arginine.

Protaminase as described in this paper does not react with more complex proteins (α-lactalbumin, β-lactoglobulin, and gelatin) and only very slightly with casein; preliminary experiments, however, have shown that a prior hydrolysis of these proteins with trypsin produces fragments which are susceptible to protaminase action. The action mechanism of protaminase on these breakdown products and the possible role of terminal arginine and lysine in this process might be of importance and requires further investigation.

Protaminase preparations described in this paper underwent marked activation upon addition of traces of trypsin (DFP added after activation). This additional activity was not due to protaminase, but rather to a new enzyme, since heat treatment of activated protaminase resulted in a "heat stable" enzyme which resembled protaminase in the sense that it hydrolyzed salmine and clupein, but did not liberate away free arginine. Similar heat treatment of protaminase itself completely destroyed the activity of the enzyme.

On the basis of the experimental evidence, it is concluded that, in addition to the known proteolytic enzymes, the glycerol extract of fresh pancreas contains an enzyme protaminase in its active form and a precursor of a "heat stable" enzyme which upon trypsin addition is converted to its active form. The fact that both of these enzymic activities are directed toward basic proteins such as protamines would indicate the importance of the basic amino acids to the specificity of these enzymes. Further investigations, however, are needed to clarify this point.

Summary

Evidence is presented that the protaminase activity occurring in pig pancreas is not associated with any of the proteolytic enzymes of this gland.

Protaminase hydrolyzed all the protamines investigated and had no activity or only a very slight activity toward the more complex proteins. The action of protaminase is confined to the liberation of free arginine from the C-terminal end of protamines. Protaminase preparations undergo activation upon addition of traces of trypsin. The observed increase in activity was shown to be due to a "heat-resistant" enzyme which, in its inactive form, accompanies protaminase.

The "heat-resistant" enzyme, similarly to protaminase, is relatively

specific for protamines but is not accompanied by the liberation of free arginine.

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