Purification and Properties of Some Enzymes in Human Seminal Plasma

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Among the enzymes in human seminal plasma only the acid phosphatase has so far been extensively studied, while the proteolytic enzymes have been the subject of only few investigations. Huggins & Neal (1942) examined in some detail the fibrinolytic properties of semen and prostatic secretion, and also claimed that 'trypsin' is present in small amounts. Oettlé (1950) confirmed the presence of a fibrinolytic enzyme, for which he proposes the name 'seminin' by analogy with the plasmin of blood plasma. He also found in semen a heat-stable inhibitor of fibrinolysis. Treatment with streptokinase gave no evidence of the presence of an enzyme precursor, and furthermore he found no kinase similar to the fibrinokinase of other tissues. On the other hand, von Kaulla & Shettles (1953) reported the presence both of an enzyme precursor (similar to plasminogen) and a kinase.

Lundquist (1952) described a casein-hydrolysing enzyme which is probably identical with the 'trypsin' of Huggins & Neal. The formation of large amounts of free amino acids from a fibrin-like material produced in the vesicular glands was demonstrated. No physiological function has yet been attributed to the amino acids in semen. Evidence that at least two enzymes are concerned in this proteolytic process has been presented (Lundquist & Buus, 1952; Lundquist, 1953). Pepsinogen, or an enzyme precursor closely resembling pepsinogen, was found in human semen by Lundquist & Seedorff (1952).

In the present work we have aimed at the separation and characterization by means of low-molecular synthetic substrates of two proteolytic enzymes in semen, a proteinase and an aminopeptidase. During our work with these enzymes we have also obtained the prostate phosphatase in a state of high purity.

EXPERIMENTAL

Materials

The enzyme preparations used as starting material in all attempts at purification and separation of the proteolytic enzymes of human seminal plasma were made from batches of semen collected at a fertility clinic. Batches (50–70 ml.) of semen were received each week and centrifuged to remove cells. The plasma was mixed with 0.1 vol. 3 M acetate buffer, pH 5. Saturated $(\text{NH}_4)_2\text{SO}_4$ soln. was added to make a final

concn. corresponding to 40% saturation ($\frac{1}{3}$ vol.). After 1 or 2 hr. in the refrigerator the mixture was centrifuged. Saturated $(\text{NH}_4)_2\text{SO}_4$ was again added to the supernatant to a final saturation of 70%. The precipitate was collected by centrifuging and was sucked nearly dry on a Büchner funnel placed in the refrigerator. The material was dissolved in a small amount of water and dialysed in cellophan tubes against distilled water at 5° for 24 hr. The precipitate formed was removed by centrifuging. The volume of this enzyme preparation was generally about half that of the starting material. For storage over prolonged periods the enzyme solution was kept frozen at -20° . Some loss in proteinase activity occurred by this treatment, while peptidase and phosphatase activities remained stable.

Synthetic substrates. The following substrates were synthesized (for methods see references in parentheses): glycylglycine (Schott, Larkin, Rockland & Dunn, 1947); diglycylglycine from chloroacetylglycylglycine (Fischer, 1903, 1906); glycyl-L-leucine (Abderhalden & Weber, 1910); benzoylglycine amide (Franzen, 1909); L-leucine amide (Smith & Slonim, 1948); benzoyl-L-arginine ethyl ester and benzoyl-L-arginine amide (Bergman, Fruton & Pollok, 1939) (it was found that the yield in the last case could be nearly doubled by transforming directly, without isolation, the benzoylarginine ethyl ester into the amide); chloroacetyl-L-tyrosine (Fischer, 1904); L-tyrosine ethyl ester (Fischer, 1901).

Acetyl-L-tyrosine ethyl ester was prepared from the tyrosine ester by boiling with the equivalent amount of acetic anhydride in an excess of dry benzene. In the preparation of *N*-benzyloxycarbonyl-L-glutamyl-L-tyrosine (Bergman, Zervas, Salzmänn & Schleich, 1934), it was found advantageous to use equivalent amounts of L-tyrosine and benzyloxycarbonyl-L-glutamic anhydride in ethyl acetate for the coupling; the yield was 71%. Benzyloxycarbonylglycyl-L-phenylalanine was made as described for benzyloxycarbonyl-L-leucylglycylglycine (Schott *et al.* 1947). DL-Alanylglycine and L-leucylglycine were commercial products (Light and Co Ltd.). DL-Alanylglycylglycine was a gift from Professor Linderström-Lang, the Carlsberg Laboratories, Copenhagen. The ethyl esters of L-cysteine, *N*-phenylglycine and acetyl-DL-alanine were prepared in the usual way by dissolving in ethanol saturated with HCl and boiling for 30 min. Phenylpropionic acid (hydrocinnamic acid) was recrystallized twice for use in inhibition experiments. Melting points and nitrogen content were, for all the substances used, in agreement with the data in the literature or close to the theoretical values respectively.

Analytical methods

Measurement of the hydrolysis of casein. The procedure used has been described by Lundquist (1952).

Measurement of hydrolysis of synthetic substances. Estimations of peptidase activity were performed by titration with ethanolic HCl in acetone according to Linderstrøm-Lang (1927) and Linderstrøm-Lang & Holter (1931). The following procedure was used. 1 ml. enzyme solution is mixed with 1 ml. 0.1 M substrate solution containing 0.02 M phosphate buffer (generally a pH value of 7.7 was used). Duplicate determination of the initial titration value is performed immediately on samples taken out with a 0.2 ml. Carlsberg constriction pipette. The mixture is incubated at 37° in tubes closed with rubber stoppers. At convenient times 0.2 ml. samples are removed and the enzymic process is stopped by addition of ethanolic HCl from a calibrated Carlsberg pipette. Titrations are made from a Rehberg microburette with 0.1 N-HCl in 90% ethanol after addition of 2 ml. acetone-indicator mixture. Control experiments with enzyme without substrate and substrate without enzyme were performed frequently. These blank values were in nearly all cases insignificant. In most cases the reaction was first order and the results are expressed as the first-order reaction constant (average of three samples). Titrations with ethanolic KOH according to Grassmann & Heyde (1929) were performed in a few experiments with acetyltyrosine ethyl ester. The titrations were, however, not very satisfactory, as a high concentration of buffer had to be used, resulting in large blank values and an ill-defined endpoint because of precipitation in the ethanolic medium. We have therefore preferred a manometric method, described below, which was found to be definitely superior.

Manometric technique. The hydrolysis of the ester bond of acetyltyrosine ethyl ester and benzoylarginine ethyl ester is conveniently followed manometrically. A similar method for the determination of chymotrypsin using phenylalanine ethyl ester as substrate has recently been described by Parks & Plaut (1953). The concentrations used in our experiments were: buffer, 0.04 M-NaHCO₃; gas phase, 95% N₂ + 5% CO₂; substrate concentration, 0.05 M in the case of benzoylarginine ethyl ester and other soluble amino acid esters (L-leucine, L-cysteine ethyl ester and N-phenylglycine ethyl ester). The total volume of liquid in the Warburg vessels was 3 ml. in all cases. The substrate used for measuring chymotrypsin-like activity was acetyl-L-tyrosine ethyl ester, which is only slightly soluble in water. Kaufman, Neurath & Schwert (1949), who have established the value of this and similar esters as substrates for chymotrypsin, made their measurements in 30% methanol. In this medium the apparent enzymic activity of chymotrypsin towards benzoyltyrosine ethyl ester is approximately 10 times less than without addition of methanol. A number of our experiments were performed with the manometric technique using an overall concentration of methanol of 30% (v/v). 0.5 ml. of 0.3 M substrate solution in methanol was placed in the side arm of the Warburg vessel, the main compartment of which contained sufficient methanol to bring its final concentration to 30% (v/v). As the solubility of CO₂ in aqueous methanol is different from that in water, it was necessary to measure the flask constants in separate experiments, where known amounts of acid were added from the side arm to Warburg flasks containing NaHCO₃ in 30% methanol. The sensitivity of the method was considerably improved by using water as the solvent and having the substrate present as a fine suspension. 0.5 ml. of an aqueous suspension of the finely powdered substance (containing approximately 0.25 mole/l.) is measured into the side arm and the experiment performed in the usual

way. The velocity of the reaction is increased at least 10 times on changing from the 30% methanol medium to water. Moreover, the enzymes are more stable under these conditions and the reaction proceeds at a constant rate during the whole experiment. The reaction is followed throughout a period varying from 15 to 150 min. In all experiments with seminal enzyme preparations we have found the hydrolysis to proceed at a constant rate, but in control experiments with pure (crystalline) trypsin ('Novo') and chymotrypsin (a gift from Dr Astrup, The Biological Institute of the Carlsberg Foundation) a rapid decrease in reaction velocity was observed. This is probably due to surface denaturation in the very dilute enzyme solution. Addition of gelatin had considerable stabilizing effect, but is of course, undesirable.

Measurement of fibrinolysis. For determination of fibrinolytic activity we have employed a slight modification of the fibrin-plate method of Astrup & Permin (1947), and Permin (1950). Ox fibrinogen, purified by two precipitations with (NH₄)₂SO₄ and in some cases further purified by precipitation with an equal volume of 2 M phosphate buffer (Jacques, 1943), is coagulated in a 2.5 mm. thick layer in Petri dishes, chosen to be nearly flat. The concentration of the fibrinogen solution is 0.3% and the coagulation is brought about by addition of purified thrombin (a preparation from 'Leo' Ltd., Copenhagen). A number of holes (usually 5) are cut in the coagulum by means of a cork borer, 5 mm. in diameter, and 50 µl. of enzyme are placed in each hole. The dishes are incubated at 38° for 24 hr. and the areas of the lysed zones are measured. The dishes are photographed by placing them on photographic paper (for contact printing) and exposing them to light from an ordinary desk lamp placed vertically over the dish. This could be performed inside the incubator without unduly moving the dishes. The lysed area was measured as the difference between the products of the longest and shortest diameters (when the holes were not perfectly circular) after and before incubation. This area was found within certain limits to be nearly proportional to the enzyme concentration. An enzyme preparation contains 1 unit/ml. if the assay performed as described results in an increase in the diameter-product of 1 cm.² in 24 hr. at 38°.

As bovine fibrin contains a precursor (plasminogen) of the fibrinolytic enzyme plasmin, this kind of experiment does not decide whether a fibrinolytic enzyme is present in a sample causing lysis on the plates, or whether we have an activator which catalyses the formation of plasmin from plasminogen. In order to differentiate between these two possibilities, some experiments were performed using the technique of Lassen (1952), who found that heating of the fibrin plates to 90° for 30 min. destroys the plasminogen present in the fibrin, without seriously impeding the availability of the fibrin as a substrate for proteolytic enzymes such as trypsin.

Zone electrophoresis

Several modifications of this technique employing either filter paper or glass beads and potato starch have been tried. In the experiments with filter paper the procedure of Kunkel & Tiselius (1952) was used. Generally a few layers of Whatman no. 1 paper (40 cm. long, 8 cm. wide) were placed on top of one another, one piece was used for staining with bromophenol blue, while the others were cut in strips 1 cm. wide, and eluted for enzyme determinations. The technique

used for glass beads and starch electrophoresis was a modification of that of Kunkel & Slater (1952). A Perspex apparatus was constructed consisting of a trough of internal dimensions $40 \times 10 \times 1.5$ cm. The trough is connected through vertical slits with the solutions in the electrode vessels (cf. Fig. 1). A tightly fitting Perspex plate, greased with a little vaseline, is placed on top of the apparatus when in use.

The most effective material for supporting the buffer used in the electrophoresis was found to be potato starch, which gives rise to only very small electro-osmotic flow and hardly any 'tailing'. 500 g. of potato starch (best available commercial grade) are suspended in 5 l. of distilled water and the water is decanted after most of the material has settled. This treatment is repeated 4–5 times and followed by 2–3 similar treatments with the buffer to be used in the experiment. The starch is now suspended in sufficient buffer to make a thick paste, which is poured into the plastic trough. Before the starch is added, a Perspex plate 3 mm. thick was fixed in a vertical position about 10 cm. from the cathode end of the trough and perpendicular to the direction of the current. After settling for 10–20 min. the excess of buffer is removed from the starch mass by means of filter paper, until a hard uniform block is formed. An air space of about 2 mm. should remain between the surface of the starch and the lid. The plate is now removed cautiously, leaving a clean, dry groove in the material. A thick suspension is prepared, consisting of starch, purified as described and sucked nearly dry, and the protein solution to be examined. This suspension is poured into the slit. The whole apparatus is placed in the refrigerator (at 5°) and after equilibration for 2 hr. the electrodes are connected to the 220 v d.c. mains. The potential gradient through the starch block was usually 3 v/cm. The composition of the buffer solution has been varied, but in the most successful experiments it was as follows: amino-trishydroxymethylmethane, 0.05 M; NaCl, 0.03 M; HCl added to pH 8.1. The enzyme solutions were freeze-dried after thorough dialysis against water, and ca. 200 mg. of the dry substance were dissolved in about 2 ml. of the same buffer before mixing with dry starch. Duration of the electrophoresis, 20 hr.

For analysis, the starch block is cut into segments exactly 1 cm. wide. The cutting and transference to numbered glass tubes is performed by means of two Perspex plates 10 cm.

wide with a thin straight edge. One plate is used for cutting off the segments while the other is used for the transfer. This procedure can be made very accurate, and 5 mm. segments can easily be cut, if better resolution is desired. The segments are eluted with a suitable buffer or salt solution. Generally 10 ml. of solvent were used for 1 cm. segments. Centrifuging is not necessary, as the starch settles to the bottom of the tube quite rapidly after stirring.

Fractionation by adsorption on tricalcium phosphate gel

The procedure finally adopted in our fractionation experiments is based on the work of Swingle & Tiselius (1951). Instead of the chromatographic technique we have, however, used adsorption followed by successive elution with salt-buffer solutions, of increasing ionic strength. The $\text{Ca}_3(\text{PO}_4)_2$ gel was prepared according to MacIntire, Palmer & Marshall (1945) and used after ageing for 3 months. 80–100 ml. of enzyme solution were mixed thoroughly with an equal volume of $\text{Ca}_3(\text{PO}_4)_2$ suspension (containing 39 mg. dry matter/ml.). After centrifuging, only a small amount of protein (10–15%) and no measurable enzyme activity is present in the supernatant. The precipitate is extracted twice with 0.01 M phosphate buffer, 3 times successively with each of: 0.02 M, 0.033 M, and 0.067 M phosphate buffer, and finally 3 times with 0.067 M phosphate containing 2% NaCl. The pH of all solutions was 7.8 and 25 ml. were used each time. For extraction the precipitate is stirred vigorously with a mechanical stirrer in a plastic tube until the mixture appears homogeneous. The mixture is centrifuged for 10 min. at 3000 rev./min. During the whole procedure the temperature was kept at 5 – 10° .

Other methods

Protein determinations were made with the micro-Kjeldahl method or by measuring the optical density at $280 \text{ m}\mu$. In the latter case the specific extinction of the protein mixture (optical density/mg. N) was always determined. Phosphatase measurements were made with phenyl phosphate as substrate at pH 5.5 in citrate buffer. Incubation time, 15 min. temp., 37° . The phenol liberated was measured with phenol reagent (Folin & Ciocalteu, 1927). One unit (Gutman & Gutman, 1941) is defined as the amount of enzyme which liberates 1 mg. of phenol/hr. under these conditions.

RESULTS

In order to characterize the proteolytic enzymes in semen, a number of synthetic peptides, amides and esters were employed. Table 1 shows the results of experiments with some of the substrates used in this study. It is clear that carboxypeptidase is entirely absent, whereas peptides with a free amino group are split. The substrate specifically attacked by pepsin is not appreciably hydrolysed, though it is known (Lundquist & Sedorff, 1952) that pepsinogen does in fact occur in human semen. The reason for this absence of hydrolysis of benzyl-oxy-carbonyl-glutamyl-tyrosine is that pepsinogen is nearly quantitatively removed during the preparation of the enzyme. Moreover, the substrate used is not a very sensitive indicator for pepsin.

Fig. 1. Detail of Perspex trough for zone electrophoresis. The vertical slit, filled with cotton and buffer solution, connects the trough with the electrode vessel (not shown). Dimensions are given in text.

The activity towards the substrates for trypsin and chymotrypsin is weak or absent in the case of the amides but considerable with the esters, which are much more sensitive to trypsin and chymotrypsin respectively than the amides. Schwert, Neurath, Kaufman & Snoke (1948) state that benzoyl-L-arginine ethyl ester is hydrolysed about 60 times as fast as the corresponding amide, and Kaufman *et al.* (1949) find chymotrypsin to be more than 1000 times as active towards ester substrates than towards amides. The specificity of the substrates as indicators of hydrolysis by trypsin and chymotrypsin is, however, far from absolute.

Separation of individual enzymes. Separation by means of salt fractionation and fractionation with organic solvents at low temperatures was tried but without appreciable success. Some purification of the peptidase was achieved with acetone fractionation, but there was no clear-cut separation. The use of tricalcium phosphate gel as adsorbent, followed by elution with buffer solutions of increasing ionic strength did, however, result in considerable purification and separation of the enzymic activity towards the small peptides on the one hand, and the activity in tests with casein and acetyltyrosine ester on the other. Furthermore,

Table 1. *The activity of crude seminal enzyme towards selected synthetic substances*

Substrate	Preferentially hydrolysed by	Duration of expt.	First-order reaction const. ($10^3 k$)
Benzoyloxycarbonyl-L-glutamyl-L-tyrosine	Pepsin	18 hr.	No hydrolysis
Chloroacetyl-L-tyrosine	Carboxypeptidase	18 hr.	No hydrolysis
Benzoyloxycarbonyl-glycyl-L-phenylalanine	Carboxypeptidase	18 hr.	No hydrolysis
Benzoyl-L-arginine amide	Trypsin	18 hr.	No hydrolysis
Benzoyl-L-arginine ethyl ester	Trypsin	30 min.	16 μ mole/hr.†
Glycylglycylglycine	Peptidases	30 min.	8.3
DL-Alanylglycylglycine*	Peptidases	20 min.	28
DL-Alanylglycine*	Peptidases	60 min.	5.8
L-Leucylglycine	Peptidases	120 min.	0.66
Glycyl-L-leucine	Peptidases	150 min.	0.55
Glycylglycine	Peptidases	150 min.	0.94
L-Leucine amide	Leucylaminopeptidase	120 min.	2.5
Acetyl-L-tyrosine amide	Chymotrypsin	17 hr.	0.035
Acetyl-L-tyrosine ethyl ester	Chymotrypsin	30 min.	13 μ mole/hr.†
Benzoylglycine amide	'Endopeptidase'	18 hr.	No hydrolysis

* The reaction constants for the DL-alanylpeptides are calculated on the basis of the L-forms, as it was found that the reaction stopped completely at 50% hydrolysis.

† Manometric experiments. The reactions follow a zero-order reaction scheme.

Table 2. *Fractionation of enzyme by tricalcium phosphate adsorption*

Fraction no.	Eluent	Vol. (ml.)	Total N (mg. N/ml.)	Phosphatase (units/ml.)	Casein hydrolysis (units/ml.)	Leucine amide hydrolysis ($10^3 k$)	'Fibrinase' (units/ml.)
1	Starting material	90	1.66	4830	3.69	2.08	8.6
2	Supernatant after adsorption	140	0.219	0	0	0	0
3	0.01 M phosphate	30	0.198	0	0	0	0
4	0.01 M phosphate	16.5	0.218	0	0	0	0
5	0.02 M phosphate	25	0.239	435	0	1.61	0
6	0.02 M phosphate	26	0.285	1650	0.18	2.86	0
7	0.02 M phosphate	26.5	0.285	2340	0.19	2.36	0
8	0.033 M phosphate	26.5	0.330	3190	0.19	1.75	0
9	0.033 M phosphate	27	0.322	1995	0.21	1.35	0
10	0.033 M phosphate	29.5	0.217	1460	0.20	0.66	0
11	0.067 M phosphate	25	0.333	1165	0.52	0.52	1.8
12	0.067 M phosphate	28	0.281	1215	0.48	0.19	3.0
13	0.067 M phosphate	25	0.306	965	0.67	0.22	4.1
14	0.067 M phosphate + 2% NaCl	26	0.331	0	0.66	0.10	7.6
15	0.067 M phosphate + 2% NaCl	26	0.280	0	0.38	0.09	8.0
16	0.067 M phosphate + 2% NaCl	23	0.212	0	0.17	0.06	6.3
Recovery (% of starting material)			87	89	31	107	101
Maximum purification				3.3	None	5	5.7

a fibrinolytic enzyme, or perhaps rather an activator of plasminogen (see later), was separated from the bulk of the material, and the acid prostate phosphatase was purified quite effectively.

Table 2 shows an experiment of this kind. A number of similar experiments were done to compare the activities towards different substrates. About 20% of the nitrogen remains in the fluid after adsorption; this fluid is apparently devoid of enzymic activity. About 10% of the nitrogen adheres to the tricalcium phosphate after the final elution. The acid phosphatase was usually recovered nearly quantitatively, and the same was the case with the peptidase and the plasminogen activator; only 30–50% of the proteinase which hydrolyses casein and acetyltyrosine ethyl ester was recovered, and, moreover, the purification on a nitrogen basis of the proteinase was in most cases insignificant. Experiments of this kind, where the fractions were assayed both against casein and acetyltyrosine ethyl ester, showed complete parallelism between the two activities. In other experiments the fractions were tested against glycylglycine, glycylleucine, leucylglycine, triglycine and alanylglycine, and these activities ran parallel in all fractions, as is apparent from Fig. 2.

For further purification of peptidase, proteinase, phosphatase and fibrinase, two or three of the fractions with highest enzyme activity were combined, precipitated with ammonium sulphate, dialysed and freeze-dried. The material thus obtained was subjected to filter-paper electrophoresis. In this way the four activities could be readily separated, but very marked losses occurred, especially of the proteinase, which appears to be

Fig. 2. Activity of fractions from a $\text{Ca}_3(\text{PO}_4)_2$ adsorption towards a number of peptides. Technique as described in Experimental section. The results are plotted as first-order reaction constants. Circles correspond to the right ordinate axis, dots to the left axis. Fraction numbers have the same significance as in Table 2. The value for alanylglycine in fraction 1 is based on only one pair of samples and it may be somewhat too low.

a rather unstable enzyme. It was, however, found later in this investigation that equally good results might be obtained by subjecting the crude enzyme to electrophoresis on a starch medium without previous tricalcium phosphate fractionation. The results of an experiment of this kind are shown in Fig. 3. Three well-defined peaks were found. The first coincides with the peak of activity towards casein and acetyltyrosine ethyl ester, the second coincides exactly with the curve for phosphatase activity, but in the case of the last peak no enzymic counterpart was found. The activity

Fig. 3. Electrophoresis experiment with crude seminal enzyme. Supporting material: potato starch. Buffer: 0.05M aminotrihydroxymethylmethane (pH 8.1) + 0.03M-NaCl. Duration of experiment: 20 hr., temp. 7°. Protein was applied at the position corresponding to fraction 11. The upper curve corresponds to the left ordinate axis and gives the optical density at 280 mμ. All other curves depict the activities of the various fractions towards the substrates indicated on the curves. Casein, phenyl phosphate, glycylglycylglycine, and leucine amide correspond to the axes on the right; acetyltyrosine ethyl ester and benzoylarginine ethyl ester correspond to the axes on the left. The cathode is to the left.

Table 3. *Properties of some fractions from the electrophoresis experiment shown in Fig. 3*

Fraction number	...	...	...	...	13	17	18	20
Total N (mg. N/ml.)					0.1195	0.153	0.094	0.0765
$E_{1\text{ cm. N/ml.}}$ at 280 m μ .					8.2	9.7	—	5.82
Phosphatase activity (units/mg. N)					0	12 900	9930	0
Peptidase activity: glycylglycylglycine hydrolysis (min. ⁻¹ /mg. N/ml.)					0	2.06×10^{-2}	12.9×10^{-2}	0
Proteinase activity (units/mg. N)					5.9	0	0	0
Activity towards acetyltyrosine ethyl ester (μ mole/hr./mg. N)					15.7	0	0	0

Minimum activity of pure phosphatase, and pure peptidase calculated from fractions 17 and 18, assuming that only these two enzymes are present: phosphatase, 13400 units/mg. N; peptidase, 0.50 min.⁻¹/mg. N/ml.

Table 4. *Influence of β -phenylpropionic acid (hydrocinnamic acid) on seminal proteinase and chymotrypsin*

The results are expressed as % of the uninhibited reaction velocity. The experiment with casein was performed in the usual way; the manometric experiments with acetyltyrosine ethyl ester were performed in 0.1M-NaHCO₃, the gas phase contained 30% CO₂. Methanol not added. Concn. of hydrocinnamate, 0.08M in all experiments.

Substrate	Seminal enzyme	Chymotrypsin (0.5 μ g.)	Chymotrypsin (2.5 μ g.)
Casein	55	83	81
Acetyl-L-tyrosine ethyl ester	167	750*	100

* For interpretation of this apparent activation, see text.

towards the group of simple peptides, the maximum of which was found a little to the right of the phosphatase peak, did not correspond to any apparent peak or shoulder on the curve. The relative position of these activities is in complete agreement with the findings by paper electrophoresis. The fibrinolysin activity was not determined in this experiment, but in other similar experiments it was situated on the left part of the proteinase peak.

Table 3 shows the relative purity of the three enzyme preparations from the fractions with maximum proteinase, phosphatase and peptidase activity. On the assumption that fractions 17 and 18 contain phosphatase and peptidase as the only proteins, the specific activities of these enzymes were calculated. The turnover number for the phosphatase was calculated to be 38 000 min.⁻¹, arbitrarily assuming the molecular weight to be 100 000. The purifications attained were: for proteinase, approximately 3 times; for phosphatase, 4.5 times; and for peptidase activity, approximately 10 times. The activity of fraction 18 towards benzoylarginine ethyl ester was 0.69 μ mole/mg. N/min. Assuming that the same enzyme hydrolyses triglycine and benzoylarginine ethyl ester, the minimum activity is 2.7 μ M/mg. N/min., which is about 10 times less than the activity of trypsin.

Properties of the proteinase. In all the enzyme preparations examined so far, whether crude

ammonium sulphate precipitates or enzyme preparations purified by the various procedures described, we have always found a constant relation between the activities towards casein and acetyltyrosine ethyl ester, suggesting that the same enzyme is responsible for the two effects. As acetyltyrosine ethyl ester is characteristically hydrolysed by chymotrypsin, a comparison was made between the two proteolytic enzymes (Table 5).

The seminal proteinase has approximately the same pH optimum as chymotrypsin towards casein, but the pH/activity curve for chymotrypsin has a much broader maximum than the seminal enzyme (Northrop, Kunitz & Herriott, 1948). Benzoylarginine ethyl ester is hydrolysed very slowly by the seminal proteinase, and also by chymotrypsin as shown by Schwert *et al.* (1948). Kaufman & Neurath (1949) have found that hydrocinnamic acid is an effective inhibitor of chymotrypsin. This seems also to be the case with seminal proteinase when casein is used as the substrate. With acetyltyrosine ethyl ester no inhibition was observed with hydrocinnamate either with the seminal enzyme or with chymotrypsin. On the contrary a definite activation was observed in both cases, which was particularly marked with a low concentration of chymotrypsin (Table 4). With chymotrypsin the result may be due to a stabilizing effect of the hydrocinnamate, as chymotrypsin at the very low concentration used deteriorates rapidly. In this case then it is not possible to decide with certainty whether the addition of hydrocinnamate has any influence on the reaction velocity.

One difference between chymotrypsin and the seminal proteinase is, however, quite unambiguous, the seminal enzyme not being inhibited by diisopropyl phosphorofluoridate (DFP) at a concentration of 0.003 M, in which conditions chymotrypsin is completely inhibited. The seminal proteinase is distinguished from the so-called cathepsins by not requiring any activator, such as cyanide or cysteine, for full activity.

Properties of the seminal aminopeptidase. The enzyme which hydrolyses simple peptides such as alanyl-glycine, leucyl-glycine, glycyl-leucine, etc.,

Table 5. *Comparison between seminal proteinase and chymotrypsin*

	Seminal enzyme	Chymotrypsin
pH optimum (casein)	7.5	7-9
Hydrolysis of acetyl-L-tyrosine ethyl ester	Rapid	Rapid
Hydrolysis of benzoyl-L-arginine ethyl ester	Slow	Very slow
Coagulation of milk	+	+
Inhibition by diisopropyl phosphorofluoridate (0.003 M)	None	Complete
Cysteine (0.005 M)	No activation	No activation
Ethylenediaminetetraacetate (0.002 M)	No influence	No influence
β -Phenylpropionic acid (0.08 M)		
Substrate: casein	Inhibition	Some inhibition
Substrate: acetyl-L-tyrosine ethyl ester	Activation	(Activation?)

Fig. 4. The effect of pH on hydrolysis of L-leucine amide by crude seminal enzyme. The results are expressed as first-order velocity constants. Buffer: 0.02 M phosphate, temp. 37°.

apparently requires a free amino group. Apart from this condition we have, with the limited number of peptides available, found no special requirements regarding type or number of the amino acids present in the substrate. Among the substrates tested, alanylglycylglycine was found to be most readily attacked (Table 1). Leucine amide, which has been used as a substrate for leucine aminopeptidase (Smith & Slonim, 1948), is hydrolysed at a rate comparable with that of the other peptides used, and both glycylleucine and leucylglycine are hydrolysed.

The pH optimum for the hydrolysis of three different substrates (Figs. 4-6) was found to be around 7.8-8.0 and the general shape of the curves is similar in the three cases.

As nearly all aminopeptidases described in the literature require or are activated by various metal ions, this point was further investigated. Some illustrative experiments are collected in Table 6. No significant activation was observed with any of the metals used. The enzyme preparations as generally used were dialysed against distilled water for 30 hr. In order to remove tightly bound metals, an experiment was performed with additional dialysis against an acid buffer of pH 4.0 for a period of 30 hr. This treatment did not cause any decrease

Fig. 5. The effect of pH on the hydrolysis of glycylglycine. Conditions as in Fig. 4. Results are expressed as percentage hydrolysis.

Fig. 6. The effect of pH on the hydrolysis of glycylglycylglycine. Experimental conditions as in Fig. 4.

in enzymic activity, neither did dilution of the thoroughly dialysed enzyme with 4 vols. of water lower the velocity expressed on the basis of mg. N.

Addition of substances which bind metals through complex formation was also attempted. Cyanide and hydroxylamine had hardly any effect, but ethylenediaminetetraacetate had some effect in all cases, amounting to 50-70% inhibition, dependent upon the substrate used (Table 6). The decrease in enzymic activity is, however, small compared with the effect observed with metal-requiring peptidases.

Table 6. *Influence of metal ions on the seminal aminopeptidase*

Hydrolysis measured by acetone titrations. pH 7.8, 37°. Results are expressed as first-order reaction constants.

Enzyme preparation	Additions	Concn. (M)	10 ³ k
Substrate: L-leucine amide			
Crude enzyme	None	—	5.3
	Mn ²⁺	0.001	5.7
	Co ²⁺	0.001	5.0
Crude enzyme	None	—	3.3
	Mg ²⁺	0.001	3.1
	Zn ²⁺	0.001	2.0
	EDTA*	0.001	1.8
	EDTA	0.002	1.5
	EDTA + Mg ²⁺	0.002†	2.0
	EDTA + Zn ²⁺	0.002†	2.1
Fraction from electrophoresis	None	—	1.09
	EDTA	0.001	0.82
	EDTA + Fe ²⁺	0.002†	0.76
Substrate: glycylglycine			
Crude enzyme	None	—	1.25
	Fe ²⁺	0.001	1.11
	Mn ²⁺	0.001	1.05
	EDTA	0.001	0.47
	EDTA + Fe ²⁺	0.002†	1.12
	EDTA + Mn ²⁺	0.002†	0.72
Crude enzyme	None	—	0.88
	Co ²⁺	0.001	1.30
	EDTA	0.001	0.32
	EDTA + Co ²⁺	0.002†	0.19
Substrate: glycylglycylglycine			
Fraction from electrophoresis	None	—	3.1
	EDTA	0.001	1.6
	EDTA + Fe ²⁺	0.002†	1.1
Crude enzyme	None	—	11.3
	EDTA	0.001	3.7
	EDTA + Mg ²⁺	0.002†	2.8

* Ethylenediaminetetraacetate. In all experiments where this substance has been added with metal ions, the concentration was 0.001M. In experiments with EDTA addition the reaction rate (first-order constant) decreased during the experiment. The values listed are averages.

† Concentration of metal ions.

Table 7. *Hydrolysis of amino acid esters by seminal enzyme*

Manometric experiments in bicarbonate buffer, pH 7.7, 35°. The results are corrected for spontaneous hydrolysis.

Substrate	μmole/hr./ml.
Benzoyl-L-arginine ethyl ester	13.5
L-Leucine ethyl ester*	1.4
L-Cysteine ethyl ester	0
N-Phenylglycine ethyl ester	0
Acetyl-DL-alanine ethyl ester	0

* The spontaneous hydrolysis of leucine ethyl ester was appreciable, viz. 1.2 mole/hr./ml.

Attempts to reverse the inhibition by addition of an excess of metal ions were negative in all cases except where glycylglycine was used as substrate, where addition of ferrous iron brought about a fairly complete reversal. This observation may

suggest the presence of a certain amount of an iron-activated glycylglycine dipeptidase, but apart from this finding no experimental support for the assumption that two peptidases might be present has been found so far. The possibility that ethylenediaminetetraacetate may have an inhibitory effect *per se*, which is not due to complex formation with activating metals, should perhaps be taken into account. In these experiments the substances added (metal salts and EDTA), were incubated with the enzyme for 1 hr. before addition of substrate.

The enzyme which hydrolyses benzoyl-L-arginine ethyl ester. The enzyme in human semen which catalyses hydrolysis of the ester bond in benzoyl-L-arginine ethyl ester is apparently not identical with trypsin or an enzyme resembling trypsin, as in the fractionation experiments the esterase was completely separated from any enzyme causing casein

Table 8. *Inhibition of the enzyme which hydrolyses benzoyl-L-arginine ethyl ester and of thrombin by diisopropylphosphorofluoridate*

Enzyme	DFP (M)	Activity (μ mole/hr.)	Average	Percentage activity
1.5% soln. of freeze-dried enzyme (0.8 ml.). Buffer: 0.1M-NaHCO ₃ ; gas phase: 30% CO ₂ +70% N ₂ ; pH 7.4; 35°	0	5.53	5.43	100
		5.33		
	0.0015	2.91	2.95	54
		2.98		
Same enzyme (0.8 ml.). Buffer: 0.4M-NaHCO ₃ ; gas phase: 5% CO ₂ +95% N ₂ ; pH 7.7; 35°	0	2.74	2.82	52
		2.90		
	0	8.67	8.65	100
		8.63		
Thrombin. Buffer: 0.04M-NaHCO ₃ ; gas phase: 5% CO ₂ +95% N ₂ ; pH 7.7; 35°	0	4.45	4.46	51.5
		4.47		
	0	8.95	8.98	100
		9.00		
	0.003	0	—	0

Table 9. *Attempts to differentiate between aminopeptidase and the enzyme hydrolysing benzoyl-L-arginine ethyl ester*

The results are expressed as a percentage of the activity without any treatment.

Treatment	Benzoylarginine ethyl ester	Glycylglycyl- glycine	Leucine amide
None	100	100	100
EDTA 0.001M	100	50	50
Heating at pH 5.5 to 60° for			
8 min.	10.5	75	—
16 min.	7.6	44	—
DFP 0.003M	52	—	88

hydrolysis. Furthermore, no inhibition was observed by purified soybean inhibitor in amounts sufficient to completely inhibit pure trypsin in control experiments.

In electrophoresis experiments the esterase followed closely the aminopeptidase, so closely indeed that there seemed to be a constant relationship between the two activities, and all attempts to separate them have so far been unsuccessful.

A number of amino acid esters were tried as substrate for this enzyme (Table 7). Apart from benzoyl-L-arginine ethyl ester, only L-leucine ethyl ester was hydrolysed at a measurable rate.

Heating at pH 5.5 to 60° causes a more rapid decrease in the activity towards the benzoylarginine ethyl ester than towards the simple peptides such as triglycine and leucine amide (compare Table 9). This finding definitely points to the possibility that two different enzymes are responsible for the peptidase and the esterase effect. Experiments with DFP revealed that the esterase was inhibited to 50% both at 0.001 and 0.003M-DFP, whereas the aminopeptidase was hardly inhibited at all. In order to exclude the possibility that hydrolysis of the DFP caused a significant change in the pH,

experiments were also performed with high buffer concentration (Table 8).

Troll, Sherry & Wachman (1954) have found that plasmin hydrolyses benzoylarginine ethyl ester, and Sherry & Troll (1954) have observed that thrombin also has a very marked ability to hydrolyse this ester. In our experiments plasmin can be excluded in advance, as no trace of fibrinolytic activity was present in fractions rich in the enzyme which splits benzoylarginine ester. The hypothesis that thrombin is responsible for the hydrolyses was tested by comparing thrombin solutions with those fractions from an electrophoresis experiment displaying the highest activity in the benzoylarginine ethyl ester assay. Thrombin in amounts hardly measurable with the manometric technique still showed good activity in the clotting test with human oxalated plasma, whereas even the most active fractions of seminal enzyme did not clot human plasma. Finally, thrombin was found to be completely inhibited by DFP (Table 8). The evidence suggesting that aminopeptidase and the enzyme which hydrolyses benzoylarginine ester are different enzymes is summarized in Table 9.

The fibrinolytic enzyme. The factor in our crude enzyme preparations which causes lysis of fibrin clots both in tubes and with the plate method was considerably purified by the calcium phosphate fractionation procedure. No fibrinolytic reaction was observed when enzyme preparations (both crude and purified) were applied to fibrin plates previously heated to 90°, in order to destroy plasminogen, which is always present in fibrinogen. Control experiments with trypsin showed that the fibrin plates retained most of their susceptibility to trypsin (about 70%). This indicates that the fibrinolytic activity in our preparations is probably due to a plasminogen activator similar to fibrinokinase. The possibility should, however, be kept in mind that the heat treatment may have rendered

the fibrin coagulum resistant towards the enzyme in semen.

The site of formation of the seminal enzymes. A number of experiments have been performed with prostate and vesicular gland tissue removed at autopsy from male human corpses within 24 hr. after death. Both aminopeptidase and proteinase were found to be present in high concentrations in prostate tissue and only in small amounts, if at all, in vesicular gland extracts.

DISCUSSION

Among the enzymes studied there seems to be none that causes lysis of fibrin directly. Neither have we found any precursor of fibrinolytic enzymes, but only what appears to be an activator. The work of von Kaulla & Shettles (1953) nevertheless indicates that both a precursor, a kinase, and consequently a fibrinolytic enzyme are present in human seminal fluid. The failure to demonstrate the presence of any active enzyme may be due to the fact that all our preparations were made by ammonium sulphate fractionation. The precipitate formed at 40% saturation, which was discarded, may have contained the true fibrinase. Another possibility is that this enzyme is precipitated and consequently discarded during the dialysis against distilled water, which is also a step common to all our enzyme preparations.

The proteinase described, which was found in earlier experiments to attack fibrinogen among a number of proteins (Lundquist, 1952), may be identical with the fibrinogenase of Huggins & Neal (1942). The function of this enzyme in the ejaculate is probably to break down further the large fragments formed by the action of the fibrinolytic enzyme system. The final step in this scheme would then logically be the formation of free amino acids through the action of the aminopeptidase.

The seminal aminopeptidase does not appear to be identical with any of the peptidases described in the literature (see, for example, the review by Smith, 1951). It hydrolyses dipeptides as well as tripeptides, and shows no preference for leucylpeptides. The fact that no metal activation was observed also distinguishes this enzyme from the majority of peptidases. Certain cathepsins with ability to split small peptides do not require metal activation, but need activation by reducing substances, such as cysteine or cyanide. These substances have no effect on the seminal peptidase. However, the possibility cannot be excluded that we are dealing with two or more enzymes of very similar physicochemical properties, which we have not succeeded in separating; the fact that the relative rates of hydrolysis for a number of substrates were constant throughout all fractionation procedures speaks rather against this possibility.

Purification of prostate phosphatase has been reported by London & Hudson (1953). Their method appears to be rather laborious, involving fractionation with acetone, ammonium sulphate, calcium phosphate absorption and 'foam fractionation'. They claim to have found a turnover number for their purest preparation of 100 000. A simple and apparently very efficient method for the purification of this enzyme by chromatography on ion-exchange resins has recently been described by Boman (1954).

Roche & Bouchilloux (1953) have reported the preparation of electrophoretically homogeneous alkaline intestinal phosphatase. From their results a turnover number of 23 000 is calculated, based on an assumed molecular weight of 100 000. They use glycerophosphate as substrate, and when it is taken into account that phenylphosphate is hydrolysed about twice as rapidly (King & Delory, 1939), the turnover number found by the French workers is not very different from that calculated for our seminal enzyme preparations. This finding may suggest a considerable similarity in the catalytic activity of acid and alkaline phosphatases.

SUMMARY

1. By means of various fractionation methods, including adsorption on tricalcium phosphate gel and zone electrophoresis on starch, the separation of at least two proteolytic enzymes from human seminal plasma was accomplished.

2. One of these is a proteinase with a certain resemblance to chymotrypsin. It hydrolyses acetyltyrosine ethyl ester, it does not require metal activation or activation by reducing substances. It differs from chymotrypsin in not being inhibited by diisopropylphosphorofluoridate and in the shape of the pH/activity curve.

3. The other proteolytic enzyme is an aminopeptidase with a pH optimum at 7.8 (tested with three different substrates). It does not require metal ions and hydrolyses both di- and tri-peptides. There seems to be no preference for leucylpeptides.

4. An enzyme was found which hydrolyses benzoylarginine ethyl ester very rapidly. It differs from the aminopeptidase in heat stability and in sensitivity towards diisopropylphosphorofluoridate and it is not inhibited by ethylenediaminetetraacetate. This enzyme was not separated from aminopeptidase and is not identical with trypsin, thrombin or plasmin.

5. A fibrinolytic factor, which appears to be similar to fibrinokinase, was separated from other enzymes. Considerable purification of this factor was accomplished by adsorption on tricalcium phosphate.

6. Prostate phosphatase was prepared in high purity by means of zone electrophoresis. A turnover

number of 38 000 was calculated for the purest preparation.

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REFERENCES

- Abderhalden, E. & Weber, L. E. (1910). *Ber. dtsh. chem. Ges.* **43**, 2431.
- Astrup, T. & Permin, P. M. (1947). *Nature, Lond.*, **159**, 681.
- Bergman, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
- Bergman, M., Zervas, L., Salzmann, L. & Schleich, H. (1934). *Hoppe-Seyl. Z.* **224**, 17.
- Boman, H. G. (1954). *Nature, Lond.*, **173**, 447.
- Fischer, E. (1901). *Ber. dtsh. chem. Ges.* **34**, 433.
- Fischer, E. (1903). *Ber. dtsh. chem. Ges.* **36**, 2983.
- Fischer, E. (1904). *Ber. dtsh. chem. Ges.* **37**, 2494.
- Fischer, E. (1906). *Ber. dtsh. chem. Ges.* **39**, 2931.
- Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
- Franzen, H. (1909). *Ber. dtsh. chem. Ges.* **42**, 2466.
- Grassmann, W. & Heyde, W. (1929). *Hoppe-Seyl. Z.* **183**, 32.
- Gutman, A. B. & Gutman, E. B. (1941). *Endocrinology*, **28**, 115.
- Huggins, C. B. & Neal, W. (1942). *J. exp. Med.* **76**, 527.
- Jacques, L. B. (1943). *Biochem. J.* **37**, 344.
- Kaufman, S. & Neurath, H. (1949). *J. biol. Chem.* **181**, 623.
- Kaufman, S., Neurath, H. & Schwert, G. W. (1949). *J. biol. Chem.* **177**, 793.
- Kaulla, K. N. von & Shettles, L. B. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 692.
- King, E. J. & Delory, G. E. (1939). *Biochem. J.* **33**, 1185.
- Kunkel, H. G. & Slater, R. J. (1952). *Proc. Soc. exp. Biol., N.Y.*, **80**, 42.
- Kunkel, H. G. & Tiselius, A. (1952). *J. gen. Physiol.* **35**, 89.
- Lassen, M. (1952). *Acta physiol. scand.* **27**, 371.
- Linderstrøm-Lang, K. (1927). *C.R. Lab. Carlsberg*, **17**, no. 4.
- Linderstrøm-Lang, K. & Holter, H. (1931). *C.R. Lab. Carlsberg*, **19**, no. 4.
- London, M. & Hudson, P. B. (1953). *Arch. Biochem. Biophys.* **46**, 141.
- Lundquist, F. (1952). *Acta physiol. scand.* **25**, 178.
- Lundquist, F. (1953). *Mammalian Germ Cells* (Ciba Symposium), p. 71. London: Churchill.
- Lundquist, F. & Buus, O. (1952). *Acta physiol. scand.* **25**, (Suppl. 89), 58.
- Lundquist, F. & Seedorff, H. H. (1952). *Nature, Lond.*, **170**, 1115.
- MacIntire, W. H., Palmer, G. & Marshall, H. L. (1945). *Industr. Engng Chem.* **37**, 164.
- Northrop, J. H., Kunitz, M. & Herriott, R. (1948). *Crystalline Enzymes*. New York: Columbia University Press.
- Oettlé, A. G. (1950). *Proc. Soc. Stud. Fertil.* **2**, 71.
- Parks, R. E. & Plaut, G. W. E. (1953). *J. biol. Chem.* **203**, 755.
- Permin, P. M. (1950). *Acta physiol. scand.* **20**, 388.
- Roche, J. & Bouchilloux, S. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 567.
- Schott, H. F., Larkin, J. B., Rockland, L. B. & Dunn, M. S. (1947). *J. org. Chem.* **12**, 490.
- Schwert, G. W., Neurath, H., Kaufman, S. & Snoke, J. E. (1948). *J. biol. Chem.* **172**, 221.
- Sherry, S. & Troll, W. (1954). *J. biol. Chem.* **208**, 95.
- Smith, E. L. (1951). *Advanc. Enzymol.* **12**, 190.
- Smith, E. L. & Slonim, N. B. (1948). *J. biol. Chem.* **176**, 835.
- Swingle, S. M. & Tiselius, A. (1951). *Biochem. J.* **48**, 171.
- Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.

The Effect of *o*-Phenylenediamine on the Biosynthesis of Vitamin B₁₂: A New Vitamin B₁₂ Analogue

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By adding *o*-phenylenediamine or any one of a number of related substances to a synthetic medium, Dulaney & Williams (1953) claimed to have doubled or trebled vitamin B₁₂ production by *Streptomyces griseus* and some other species, as judged by the *Escherichia coli* (mutant) assay. 1:2-Diamino-4:5-dimethylbenzene, a part of the vitamin B₁₂ molecule and a postulated precursor of it (Woolley, 1950, 1951), had no such effect. These authors thought it unlikely that increased vitamin

B₁₂ was produced by incorporating phenylenediamine into the molecule. Tracer studies with an unstated level of deuterio phenylenediamine showed that not more than 20% of the vitamin was labelled. It was shown that phenylenediamine inhibited the growth of *Strep. griseus* and it was suggested that a compound antagonistic to phenylenediamine, namely vitamin B₁₂, was produced in increased amounts so as to overcome this inhibition.