PROTEASES FROM MOUSE SUBMAXILLARY GLAND

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SUMMARY Two proteases ("A" and "D") from mouse submaxillary gland have been isolated in high purity. At pH 8.5 "D" is more negative than "A" and is also smaller. Amino acid composition differs only in 6 constituents. "D" is activated 100-300% when acting on tosyl arginine methyl esther by tosyl arginine and all α-amino acids. Both enzymes have a strong preference for arginyl bonds.

We recently reported an improved method for the isolation of Nerve Growth Factor (NGF) with very high specific activity and unusual stability (1,2). Further development of this method allows the separation of four estero-proteolytic enzymes which are devoid of NGF activity. Their designation in this report as "A", "B", "C", and "D" relates to their electrophoretic mobilities on analytical acrylamide gel columns (Fig. 1). We have isolated two of these, "A" and "D" in high purity. Both show a marked degree of activity toward α N, substituted arginine esters. A number of protein subtrates have been studied and here too it appears that the peptide bonds involving arginyl carboxyl groups are the most susceptible ones. Enzyme "D" is activated by all α-NH_2 acids.

EXPERIMENTAL The method of their isolation is as follows: Two thousand submaxillary glands from adult male mice (over

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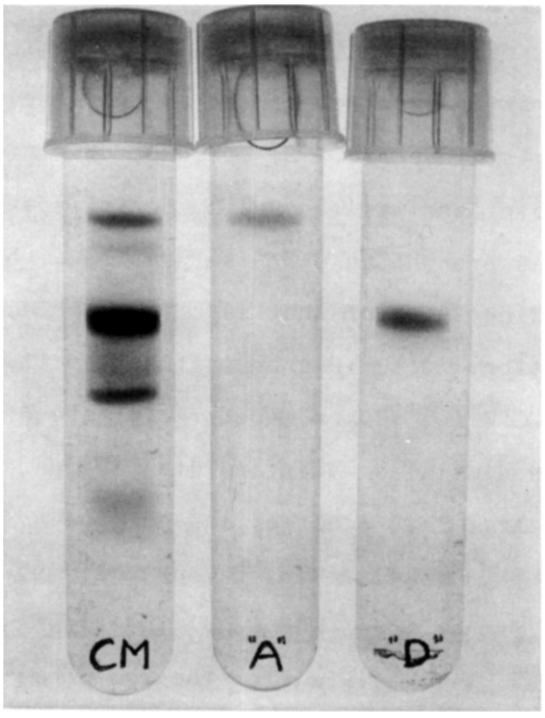

Figure I. Analytical acrylamide gel electrophoresis of the fraction from carboxy methyl column chromatography and enzymes "A" and "D" after preparative acrylamide gel electrophoresis.

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30 gm) obtained frozen from Pell-Freez Biologicals, were thawed and homogenized in a Waring blendor at maximum speed, with one liter of 0.1 M phosphate buffer at pH 7.4. The preparation was centrifuged at 10.000xG for 10 min and the precipitate discarded. One volume of 0.2 M streptomycin sulfate (adjusted to pH 7.4 with NaOH) was added to each nine volumes of extract. After 3 hr at 3-5°, the precipitate was removed by centrifugation. Seven ml of cold absolute ethanol was slowly added to each 100 ml of the supernatant. After 15 min at 0-4° the mixture was centrifuged. The pellet was discarded and 48 ml of ethanol (-15°) added to each 100 ml of the streptomycin supernatant. After 2 hr at -15°, centri-

fugation gave a gummy red precipitates. This was disolved in 500 ml of cold water with stirring. The solution was brought to 35% saturation with respect to ammonium sulfate by adding 24.5 gm of the solid per 100 ml of solution. After 1 hr at 3-5°, the precipitate was removed by centrifugation. The supernatant was brought to 85% saturation with ammonium sulfate by adding 35 gm of that salt for each 100 ml of original solution. After 2-4 hr in the cold it was centrifuges at 15,000xG for 30 min, the precipitate disolved in 100 ml of cold distilled water and dialyzed until free of ammonium sulfate. This fraction was applied to a 2.5x60 cm column of carboxy-methyl cellulose, prepared by the method of Cohen for the isolation of NGF (3). The flow rate was kept below 3 ml/min. Virtually all of the red pigment was retained by the cellulose, but neither NGF nor the estero-proteolytic enzymes were held. They were quantitatively recovered with 2-3 column volumes of water. This eluate was lyophilized, the residue disolved in a small volume of water, and exhaustively dialyzed against a 0.05 M Tris HCl buffer at pH 7.5. A portion of the dialysate (200-500 mg of protein) was applied to a 2.5x 165 cm column of DEAE-Sephadex A-25 (Pharmacia) prepared as in method "B" given by the manufacturer (4). The column was thoroughly equilibrated with the Tris buffer. Elution was carried on at 250 ml/hr to obtain the pattern of separation shown in Fig.II. Peak II contained a mixture of the enzymes of interest. Peak V contained the NGF activity.

Further purification of enzymes "A" and "D" was accomplished by preparative acrylamide gel electrophoresis using the "Fractophorator" described in Ref.5. Table I shows the yields obtained for the various steps of the isolation.

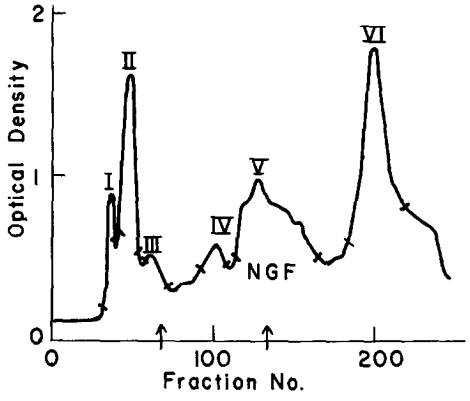

Figure II. DEAE-Sephadex column chromatography described in text. The arrows are points of addition of 0.1 M NaCl and 0.2 M NaCl in the buffer respectively.

TABLE I

Fraction		Protein		Esterase		Specific activity	
Crude homogenate		24.800	mg	3.65×10^6	units	150	U/mg
85% ammonium sulfate		3680	"	2.66×10^6	"	725	"
Carboxy methyl fraction		2362	"	2.32×10^6	"	1,000	"
DEAE-Sephadex peak II		400	"	7.2×10^5	"	1,800	"
Preparative	"D"	174	"	$6.9\text{-}10.4 \times 10^4$	"	400-600	"
Electrophoresis	"A"	86	"	2.6×10^5	"	3,000	"

(1) The enzyme unit is defined as the amount of protein that hydrolyses 1 μ M of TAME/min at pH 8.0 and 37°. The "no enzyme" rate under these conditions is about 0.015 μm/min and is subtracted.

The reaction mixture consists of 3 ml of solution which is 0.1 M in Kcl, 0.0053 M TAME and 0.0026 M glycine. The reason for the presence of glycine is discussed in the text.

(2) The total esterase units of the crude homogenate reflect the combined action of a variety of enzymes in the mouse submaxillary gland (e.g., Kallikrein) that act on the substate (TAME) that served for the definition of the enzyme unit. These various enzymes have different specific activities and this must be taken into account in the evaluation of the table of yields.

(3) We have observed considerable variability in total protein as well as esterase units in these preparations. We ascribe it to the age of the submaxillary glands used as starting material.

Figure I includes the analytical acrylamide gels (6) (stained with amido black) of the enzymes after the last step described in the isolation procedure. At levels of 100-150 µg of protein added to the gels, both enzymes "A" and "D" ran as single bands indicating a high degree of purity.

TABLE II

Amino Acid Composition of Enzymes "A" and "D"

	Residues/M analytical "A"	"D"	Residues/M integers "A"	"D"
Aspartic acid	30.8	30.9	31	31
Threonine	15.4	16.3	15	16
Serine	18.2	17.8	18	18
Glutamic acid	21.8	21.9	22	22
Proline	20.1	16.0	20	16
Glycine	33.0	32.2	33	32
Alanine	14.8	14.8	15	15
Cysteine	9.4	8.6	9	9
Valine	13.7	9.1	14	9
Isoleucine	11.7	5.9	12	6
Leucine	27.5	25.1	27	25
Tyrosine	7.5	9.0	8	9
Phenyl alanine	8.2	8.2	8	8
Methionine	5.4	[4.0]	5	4
Lysine	21.5	22.0	22	22
Histidine	6.7	7.3	7	7
Arginine	[4.0]	4.85	4	5
Tryptophan (from U.V. absorbance)	12.0	7.0	12	7

Residue wts. 30,643 ("A"), 28,330 ("D")

The results given are the averages of 5 analyses of each enzyme using hydrolysis times of 24, 48, and 72 hr. The M ratios were calculated using the parenthetical figures as guides.

Table II shows the amino acid content of the enzymes after acid hydrolysis. There is a remarkable similarity of composition between "A" and "D". Significant differences are found

only in 6 amino acids. Summation of the found residue-weights gives a molecular weight of about 30,000 for enzyme "A" and 28,000 for "D" in good agreement with the estimates of their molecular weights based on the observed S_{20} values of 3.1 S and 2.8 S respectively.

Rabbit antiserum to the material in peak II of the DEAE-Sephadex column was prepared. Enzymes "A" and "D" were found to be immuno-electrophoretically pure and distinct, but antigenically related (7). The antiserum abolished the enzymatic activity of both enzymes. It does not affect the action of trypsin.

TABLE III

Hydrolyzed	Not hydrolyzed
Tosyl arginine methyl ester	Leucine ethyl ester
Benzoyl arginine ethyl ester	Lysine methyl ester
Polyarginine	Poly lysine
Cytochrome "C"	Arginyl-glycyl-glycine
Lysozyme	Alanyl glycine
Arginine and lysine rich histones	Glycyl alanine
	Glycyl proline
Protamine sulfate	Glycyl leucine
	Valyl glycine
	p-nitro-phenyl acetate
	Glycyl-Glycyl alanine

Table III shows the various substrates that have been assayed with the enzymes. Hydrolysis was followed by measurement of alkali uptake using a pH stat (Radiometer Copenhagen) set at pH 8.0 or by formol titration (9). For rapid scanning of enzymatic activities the change in color produced by hydrolysis of an alkaline solution of p-toluene sulfonyl arginine methyl ester (TAME) in the presence of phenol red was used.

Formol titrations indicated that polyarginine (M.W. about

150,000) was rapidly hydrolyzed to di- and tri-arginine peptides, with some release of free arginine, while poly-lysine (M.W. 150-200,000) was not attacked at all. The extent of hydrolysis of protamine and of arginine or lysine rich histones also correlated well with the arginine content of these proteins and yielded peptides with C-terminal arginine. Addition of trypsin to the lysine rich histones after hydrolysis with enzymes "A" and "D" or a mixture of both, gave a formol titer consistent with the additional splitting of the lysine bonds.

Figure III shows the time course of hydrolysis of TAME by enzymes "A" and "D". The sigmoid nature of the curve for enzyme "D" is abolished upon addition of p-tosyl arginine to the reaction mixture, resulting at the same time in a significantly enhanced initial rate. Similar additon of methanol, the other product of the reaction, has no effect. This activation by a product of the hydrolysis led to the examination of the effect of free amino acids. It was found that all amino acids are activators to varying degrees. Enhancements of rate of hydrolysis range between $100^0/_0$ and $300^0/_0$. Several D-amino acids tested (alanine, glutamic acid, and phenylalanine) were as effective as the corresponding L-isomers.

Comparison of sodium acetate, acetamide, and glycine indicated that only the latter activated. Enzyme "A" is not activated by either amino acids or tosyl arginine.

The K_m's for the hydrolysis of TAME at pH 8.0 were determined for both enzymes. For enzyme "A" the value is 6.6×10^{-4} M/l at 37^0. For enzyme "D" (activated with glycine) the values obtained range from 2.4 to 3.1×10^{-4} M/l at 37^0,

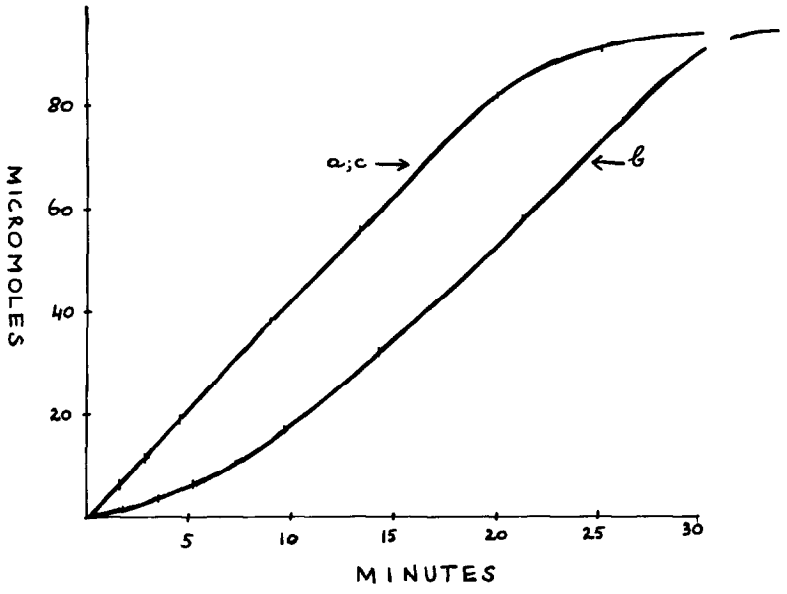

Figure III. Time course of action of enzymes on TAME. (3 ml of 2.3×10^{-3} M) a. Enzyme "A" with or without tosyl arginine. b. Enzyme "D" without activator. c. Enzyme "D" with 1.2×10^{-3} M tosyl arginine initially present.

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because of some uncertainty in the interpretation of Lineweaver-Burke plots. A comparison of the specific activities of these enzymes towards TAME and similar esters with that of trypsin shows that they are from 2-15 times more active. They appear less active with protein as substrates though with protamine or polyarginine the extent of hydrolysis eventually reaches the same point as that reached by trypsin.

Inhibition studies on enzyme "D" have been done with various agents. At concentrations ranging from 1×10^{-2} M to 1×10^{-5} M of Fe^{+++}, Cu^{++}, Zn^{++}, Cd^{++} the enzyme was inhibited from 50% to 100%. EDTA can restore up to 85% the activity of the Cu^{++} inhibited enzyme. A plot of return of enzyme activity versus EDTA concentration at fixed TAME and

Cu^{++} concentration showed a sigmoid curve. Mercaptoethanol (0.5 to 3.0%) had no effect on the rate of hydrolysis of TAME or of protamine sulfate. Urea (4-10 M) in the substrate solution inhibits the enzyme. The amount of substrate hydrolyzed when enzyme stopped acting as a result of urea, was smaller, the higher the urea concentration.

DISCUSSION There have been earlier reports on the presence of estero-proteases in the mouse submaxillary gland. Attardi et al. (10) have reported on two enzymes, one of which caused demonstrable dedifferentiative changes in muscle tissue from 11 day old chick embryos cultured in vitro, but showed scant proteolytic activity on denatured casein. Proteolytic activity for the other was not reported. More recently, Calissano and Angeletti (11) have studied the effect of androgens on the biosynthesis of two estero-proteases from the mouse submaxillary gland. These enzymes were both inactivated by mercaptoethanol. Since bond specificity with protein substrates or kinetic and physico-chemical characteristics have not been reported for either group of enzymes, it is not clear whether the same or different proteins are involved.

The characteristics of the enzymes reported on here distinguish them clearly from trypsin. Immuno-electrophoretic studies as well as a comparison of their amino acid content seems to point to a structural relatedness. "D" is smaller than "A" and differs in composition by relatively few amino acid residues as if limited proteolysis converted "A" to "D".

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REFERENCES

1. Schenkein, I., Levy, M., Bueker, E.D. and Tokarsky, E., Science, 159, 640, 1968.
2. Schenkein, I., Bueker, E.D. and Levy, M., Conference on Hormones in Development. 1968, Nottingham, England (in press).
3. Cohen, S., Proc. Nat. Acad. Sci. (US), 46, 302, 1960.
4. Pharmacia Fine Chemicals Inc. Sephadex ion exchangers, undated.
5. Schenkein, I., Levy, M., and Weis, P., Analytical Biochem., 25, 387, 1968.
6. Davis, B.J. and Ornstein, L., Ann. N.Y. Acad. Sci., 121, pp. 321, 404, 1964.
7. Scheidegger, J.J., Inter. Arch. Allergy Appl. Immunol., 7, 103, 1955.
8. Baines, N.G., Baird, J.B. and Elmore, D.T., Biochemical J., 90, 470, 1964.
9. Levy, M., IN: Methods in Enzymology, (S.P. Volowick and N.O. Kaplan, Eds.), vol. 3, 454, 1957.
10. Attardi, D.G., Schlessinger, M.J. and Schlessinger, S., Science, 156, 1253, 1968.
11. Calissano, P. and Angeletti, P.U., Biochem. Biophys. Acta, 156, 51, 1968.