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TESTOSTERONE INDUCTION OF ESTERO-PROTEOLYTIC ACTIVITY IN THE MOUSE SUBMAXILLARY GLAND

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SUMMARY

Testosterone injections sharply increase the estero-proteolytic activity in submaxillary gland extracts of female and of adolescent mice. A major form of hormone-dependent enzyme has been isolated and purified from male gland extracts. The enzyme has a molecular weight of about 32 000 and appears to be a peptide hydrolase with strong esterolytic activity. A specific antiserum to the purified enzyme was prepared and used for immunochemical titrations. The results indicate that the increased enzyme activity in the gland following testosterone treatment is due to an increased concentration of enzyme molecules. Incorporation studies gave additional evidence that the hormone stimulates the rate of the enzyme synthesis in the gland.

INTRODUCTION

The sexual dimorphism of the mouse submaxillary gland is reflected in marked differences at the biochemical level[1-3]. The secondary sex differences appear to be restricted to the convoluted tubular portion of the gland and a high degree of specificity exists with respect to the control exerted by androgenic hormones. Our interest in the sexual dimorphism of the mouse submaxillary gland arose from the finding that a specific nerve-growth factor (NGF) is present in very high concentration in the adult male glands and about 10 to 20 times less in the respective female glands[4]. The NGF content as well as the protease–esterase activities of the gland extract are strikingly increased by testosterone treatment of female and of prepuberal mice, while other enzyme activities such as amylase are practically unaffected[5]. The parallelism between NGF activity and esteroproteolytic activity in the submaxillary gland prompted us to investigate further the mechanism by which the activity of these proteins is stimulated by male hormones. The observed increase of biological activities in the gland extracts could in fact be explained by several alternative mechanisms such as increas-

* Abbreviations: BANA, α-*M*-benzoyl-DL-arginine-β-naphthylamide; BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; NGF, nerve-growth factor.

ed availability of activators or removal of inhibitors, altered intracellular distribution, conformational changes and increased level of active protein molecules due either to increased synthesis or to slower breakdown. In the present paper, data concerning the induction of an enzyme activity in the gland are reported. A major form of testosterone-inducible estero-peptidase was isolated and purified from the gland extracts. Specific antibodies to the purified enzyme were then used for immunochemical titration of the enzyme in extracts of normal and hormone-induced animals.

MATERIALS AND METHODS

Mice of Swiss strain were used in all experiments. The animals were all fasted for 5 h before the sacrifice. Submaxillary glands were quickly removed and dissected out from adipose and lymphatic tissue and frozen in dry ice. Homogenization was done in a glass homogenizer or in a 'Virtis' homogenizer, in 5 mM Tris buffer (pH 7.4). The gland homogenates were all centrifuged at 10 000 rev./min for 30 min in a refrigerated centrifuge (0–5°) and the clear supernatant used for all the biochemical and immunological experiments.

Testosterone proprionate (Vister) was injected either subcutaneously or intraperitoneally in the amount of 0.1 mg in prepuberal mice about 20 days old, or 1 mg in adult female mice.

Substrate and enzymes

α-*N*-Benzoyl-DL-arginine-β-naphthylamide (BANA), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), α-*N*-benzoyl-L-arginine ethyl ester (BAEE), *N*-acetyl-L-tyrosine ethyl ester (ATEE), L-leucyl-β-naphthylamide, α-naphthyl acetate, β-naphthyl acetate, α-naphthyl butyrate were from Sigma Chemical Co. (St. Louis). Solutions of various substrates were freshly made, dissolved in buffer either directly or after dissolution in a proper solvent (dimethylsulfoxide or acetone). Crystallized trypsin, crotalus atrox, ovomucoid, soybean, DFP, *p*-chloromercuribenzoate, mercaptoethanol were from Sigma Chemical Co. Trasylol was a commercial preparation from Bayer (Germany).

Enzymatic assays

Incubations were carried out in Tris buffer (pH 8–10) or phosphate buffer (pH 6–8).

Proteolytic activity on casein was assayed according to KUNITZ.

Hydrolysis of substrates yielding 2-naphthylamine or naphthol was measured using diazo-coupling according to HOPSU AND GLENNER[6].

Proteins were measured by the method of LOWRY *et al.* using bovine serum albumin as standard[7].

Chromatography and electrophoresis

DEAE-cellulose and CM-cellulose were from Sigma Chemical Company. Sephadex G-100, commercial preparation, was from Pharmacia (Sweden). Chromatography and gel filtration were carried out at 4°.

Vertical starch-gel electrophoresis and disc electrophoresis on acrylamide gel were carried out as described in the RESULTS.

Radioactivity counting

Incorporation studies were carried out by incubating tissue slices in Warburg vessels in the presence of radioactive amino acid mixture (New England Corporation). Radioactivity in the various samples was measured in a liquid scintillation system (Tri-Carb-Packard).

RESULTS

In a first series of experiments, protein extracts from normal male and female

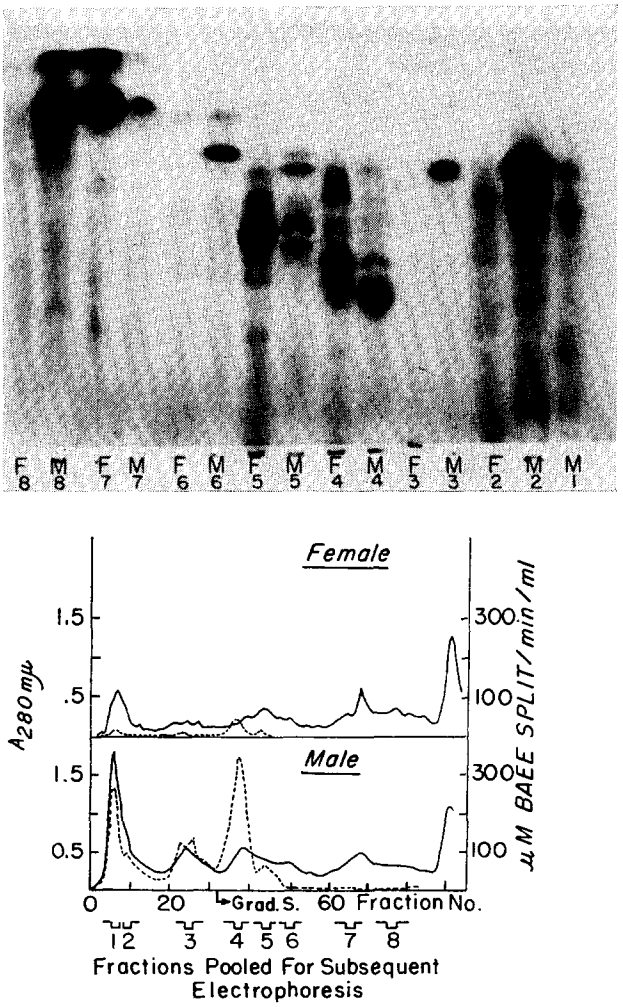

Fig. 1. Chromatographic patterns of soluble proteins from male and female mouse submaxillary glands (50-day-old animals). ———, protein; — — —, esterase activity. Identical DEAE-cellulose columns, equilibrated with 5 mM Tris-HCl buffer, were used; a gradient salt elution from 0 to 1 M NaCl was simultaneously applied to the two columns. Corresponding chromatographic fractions from the male and the female patterns were pooled together and fractionated further on vertical starch-gel electrophoresis (top figure).

glands were fractionated by column chromatography on DEAE-cellulose; the main chromatographic components were further fractionated by starch-gel electrophoresis. The combined use of these fractionating procedures allowed a greater resolution of the total soluble protein (Fig. 1). When the male and female patterns were compared, a number of quantitative as well as qualitative differences were observed in the distribution of various protein fractions. As can be seen, the esterase activity was separated into four distinct components in the chromatogram. In the male pattern over 60% of the total applied BAEE-splitting activity was recovered in a sharp peak emerging soon after the beginning of the gradient. The relative amount of this enzyme component was 20 times higher in male than in female glands (Figs. 1 and 3). Further purification of this enzyme activity was achieved by gel filtration and column chromatography (Fig. 2).

On both starch-gel and acrylamide electrophoresis, the Sephadex fraction showed two distinct components, both endowed with BAEE-splitting activity, al-

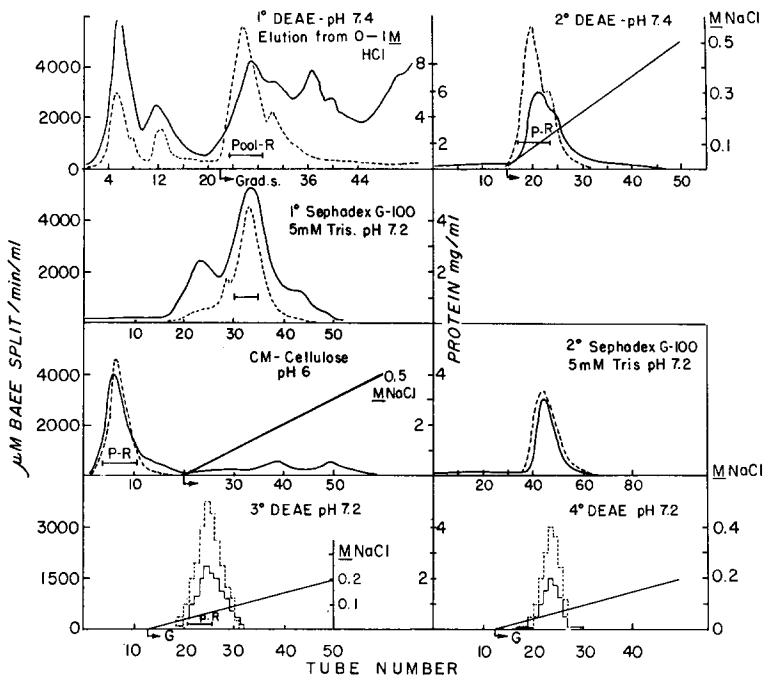

Fig. 2. Purification of the main post-gradient estero-protease (pool R) through ion-exchange chromatography and gel filtration. ———, protein; — — —, esterase activity.

though the specific activity of the slower moving one was 2 times higher than the other. When the other of the two closely related isozymes could be separated on a DEAE-cellulose column at pH 7.2, using a shallow salt gradient from 0 to 0.2 M NaCl, the esterase with the highest specific activity was then chosen for further characterization and for the preparation of specific antiserum. The final preparation showed one single component on disc electrophoresis (Fig. 4). The molecular weight was calculated to be approximately 32 000 by using Sephadex filtration and centrifugation on sucrose

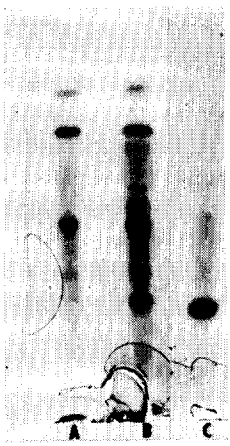

Fig. 3. Starch-gel electrophoresis of male (A) and female (B) submaxillary gland extract and of the partially purified R-esterase (C) (DEAE fraction).

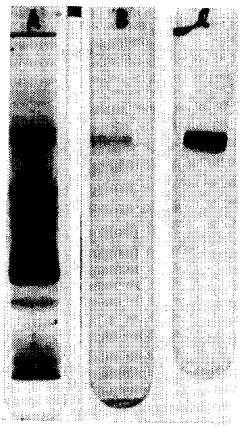

Fig. 4. Disc electrophoresis on acrylamide gel (glycine–NaOH buffer (pH 8.3) of (A) crude submaxillary extracts, (B) purified R-estero-protease (15 μg) and (C) the same, 100 μg.

gradient. The enzymatic activity was tested with various substrates and compared with that of trypsin and of snake venom.

As shown in Table I, the purified submaxillary enzyme hydrolyzes at a very fast rate BAEE and some other esters; it also shows a limited proteolytic activity when tested with casein or hemoglobin. The synthetic endoprotease substrate BANA was hydrolyzed 20 times slower than by trypsin, while BAPA was hydrolyzed to the same extent. As shown in Table II, trypsin inhibitors soybean and ovomucoid have no effect on the enzyme activity, which was instead strongly inhibited by trasylol, mercaptoethanol, and by organophosphorus compounds. The pH optimum of the enzyme was found to be, with various substrates, in the alkaline range, between 8.8 and 9.2. It appears therefore that the purified submaxillary enzyme acts as an alkaline peptide-hydrolase with a very strong esterolytic activity.

TABLE I

HYDROLYSIS RATE OF VARIOUS SUBSTRATES BY SUBMAXILLARY ENZYME AND BY TRYPSIN
Tris–HCl buffer, pH 8 (trypsin) and pH 9 (submaxillary enzyme).

Substrate	*Trypsin*	*Submaxillary enzyme*
Casein (Kunitz units/mg)	5.8	0.16
BANA μmoles/min per mg	0.85	0.05
BAPNA μmoles/min per mg	3.6	4.2
BAEE μmoles/min per mg	64	1500
α-*N*-Benzoyl-L-Arginine methyl ester μmoles/min per mg	31	1100
ATEE μmoles/min per mg	0.8	27
Leucylnaphthylamide μmoles/min per mg	0	0
β-Naphthyl acetate μmoles/min per mg	0	1.65
α-Naphthyl acetate μmoles/min per mg	0	2.5
α-Naphthyl butyrate μmoles/min per mg	0	1.8

TABLE II

EFFECT OF VARIOUS COMPOUNDS ON THE HYDROLYSIS OF BAEE BY THE SUBMAXILLARY ENZYME

Compound	*Concentration*	*Inhibition (%)*
Ovomucoid	0.1 mg/ml	0
Soybean	0.1 mg/ml	0
Trasylol	5 I.U./μg enzyme	80
EDTA	2 mM	0
Mercaptoethanol	0.4 mM	80
Diisopropylfluorophosphate	1 μM	100

5 mg of this enzyme preparation were then used to immunize a rabbit with the aid of Freund's complete adjuvant. The antiserum thus obtained was used to run a quantitative precipitin reaction. As shown in Fig. 5, the enzyme activity could be quantitatively recovered in the specific precipitate redissolved in 5 mM NaOH. The precipitin curve appeared consistent with a single antigen–antibody system and the point of maximum protein precipitate coincided with the maximum enzymatic activity. One single band of precipitation was obtained on Ouchterlony plate and on immunoelectrophoresis.

The antiserum was tested with crude submaxillary gland extracts and with mouse kidney and liver extracts. As shown in Table III, no cross reaction was observed with mouse kidney or liver. With the submaxillary gland extracts from both male and female mice, a specific precipitate was obtained which seemingly showed a specific activity of about 1000 moles BAEE/min per mg protein. Since the specific activity of the pure antigen is about 1500 moles BAEE/min per mg, this finding suggests that the antiserum would cross-react with some other protein in the crude submaxillary extracts, possibly with some of the isozymes with lower specific activity already shown to be present there. Indeed, in preliminary experiments with two other

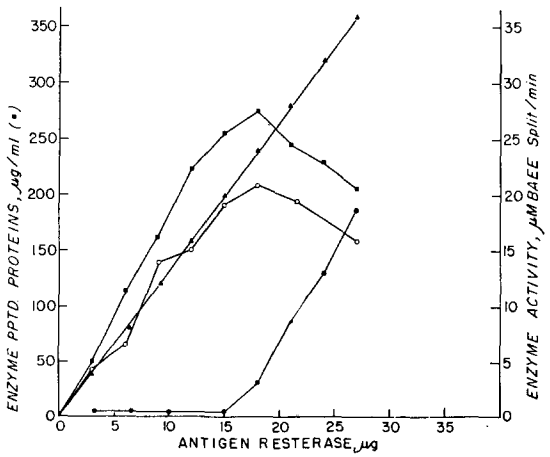

Fig. 5. Precipitin-curve of the purified R-enzyme and its antiserum. ■—■, protein precipitated; ○—○, enzyme activity recovered in the precipitate; ●—●, enzyme activity in the supernatants; ▲—▲, enzyme activity (μmoles/min per ml) added.

TABLE III

IMMUNOCHEMICAL ASSAY OF THE SUBMAXILLARY ENZYME IN VARIOUS TISSUE EXTRACTS IN MICE

Tissue	*μg of enzyme precipitated per mg total soluble protein*
Kidney	<1
Liver	<1
Adult male submaxillary gland	115–135
Adult female submaxillary gland	5–7
16-days mouse submaxillary gland	<1

purified BAEE-splitting enzymes, chromatographically and electrophoretically distinct from our enzyme, the antiserum was seen to cross-react to the extent of 15% and 70% respectively.

As seen in Table III, the actual concentration of the enzyme shown by the immunochemical assay was 20 times higher in the male extracts than in the female. After testosterone treatment, the increase of the enzyme activity in the female glands was found to be strictly proportional to the increase of the precipitable enzyme present in the extracts. Similar results were obtained in the adolescent mice (18-days old) induced with a single injection of the male hormone (Fig. 6). As can be seen, practically no precipitate is obtained with gland extracts for the first 2 weeks after birth. At 18 days, in both male and female mice, the enzyme content of the gland is less than 0.1% of the total soluble protein. A single injection of testosterone at this age brings about a striking increase of the enzyme content in the gland, which after 4 to 5 days is up to 3% of the total proteins.

From the immunochemical data, the increase of the enzyme activity appears

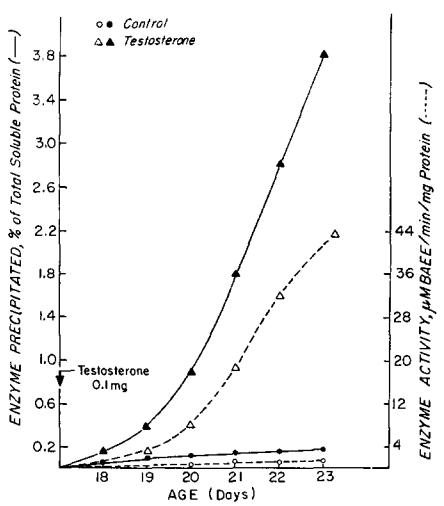

Fig. 6. Immunochemical assay of the estero-protease in the mouse submaxillary gland after testosterone stimulation. Each value is the mean of three separate determinations.

entirely due to an actual net increase of the enzyme-protein concentration. Such a dramatic increase could hardly be explained by a reduced rate of enzyme breakdown as a consequence of the hormone action, but more conceivably appears due to an increased rate of enzyme synthesis.

That this was the case was further confirmed by incorporation studies. Slices of submaxillary gland from 30-days old normal female and from testosterone-treated litter mates were incubated in vitro for 30 and 60 min at 38° in a medium containing a radioactive amino acid mixture. At the end of the incubation period, the tissue slices were completely washed in a cold medium and then homogenized in Tris–HCl buffer, 5 mM (pH 7.2). The homogenate was centrifuged at 10 000 rev./min; aliquots of the clear supernatant were precipitated with cold 5% trichloroacetic acid for counting of the radioactivity in the total soluble protein. Other aliquots were precipitated with the antiserum in the region of antibody excess. These antiserum precipitates were washed 3 times with saline and finally dissolved in 5 mM NaOH for assay of protein and esterase activity. The remainder was precipitated again with trichloroacetic acid. All of the trichloroacetic acid precipitates were washed several times and finally the radioactivity was counted in a liquid scintillation system.

TABLE IV

RADIOACTIVE AMINO ACID INCORPORATION INTO SUBMAXILLARY ENZYME IN NORMAL (N) AND TESTOSTERONE-TREATED (T) FEMALE MICE

Status	*Total soluble protein (mg/ml)*	*Enzyme activity (μmoles BAEE/min mg)*	*Counts/ min in enzyme precipitated per ml*		*Counts/min per ml total soluble protein*	
			30 min	*60 min*	*30 min*	*60 min*
N	3	1.0	180	340	3250	7050
T	3	1.6	650	1360	4180	8850
T/N ratio		1.6		4.0		1.25

Table IV shows that the incorporation rates increased linearly with time. The antiserum precipitates showed a higher rate of incorporation as compared to the total soluble protein, thus reflecting a higher synthetic rate of this secretory enzyme. After testosterone treatment (48 h), the incorporation rate appeared to increase about 30% in the total soluble protein but a 3- to 4-fold increase of the incorporation rate in the enzyme precipitated was observed.

DISCUSSION

There is a large body of evidence that androgen stimulation of sex-specific and sex-non-specific tissues can influence effective enzyme concentrations in these tissues[8–11]. Hormonal control of proteolytic activity in the mouse submaxillary gland was first described by JUNQUEIRA and co-workers[2,12] and later confirmed by others in mice as well as in rat[13,14]. Another interesting aspect of the sexual dimorphism of the mouse submaxillary gland is the observed androgen-dependent concentration of a

specific protein endowed with growth promoting activity on some nerve-cell types, the NGF. The striking parallelism between the testosterone induction of NGF and esteroproteolytic activity of the gland first focussed our attention on the mechanism by which androgen hormones can regulate the effective concentration of these proteins in the gland. The results reported in the present paper are dealing with an increase of the esterase activity after testosterone stimulation of female and of prepuberal mice. A major form of trypsin-like esterase, with a pH optimum in the alkaline range, has been purified and partially characterized. The enzyme exerts a hydrolytic activity on several peptides as well as ester bonds. It appears to be quite different from trypsin and chymotrypsin, plasmin and thrombin, because of its pH optimum, inhibitor effects and substrate specificity. It also appears to be diffierent from salivary kallikrein, for its ability to split endoprotease substrates such as BANA and BAPA is far greater.

The enzyme appears to be related to the digestive functions of the gland and in normally fed male is mice present in very high concentrations in the gland extracts and in the saliva.

The actual concentration of the enzyme is markedly different in male and female glands: in the former, as determined immunochemically, it accounts for about 12–14% of the total soluble proteins; whereas in the latter, it represents only 0.5–0.7% of the total soluble proteins. Owing to cross-reaction of the antiserum with other closely related isozymes also present in the gland, these figures may be only approximately related to the actual concentration of the enzyme we have characterized. The enzyme is under control of male hormones and the sex genotype appears to have relatively little influence in the reactivity of the cells which produce it. The results of immunochemical experiments rule out the possibility that the increased enzyme activity after testosterone treatment in female and in prepuberal mice is due to a mechanism other than an increased concentration of enzyme protein. Labelling experiments give additional evidence that the enzyme protein accumulation in the gland is mainly due to an increased rate of synthesis following testosterone injection. It remains to be seen, however, if and to what extent the hormone may also affect the breakdown of the enzyme, since a slowing-down of this process could also be partly responsible for the accumulation of the enzyme within the gland cells. The above results place the action of androgen on the mouse submaxillary gland at the level of control of protein biosynthesis. The results of our fractionation studies of male and female glands indicate that a number of other proteins in addition to the NGF and the esteroproteolytic enzymes, may well be under such control. Some of these proteins which appear in markedly different concentration in the glands of different sex are now under investigation. The site at which this control is exerted remains to be seen. Preliminary experiments now under way indicate that testosterone may increase the rate of synthesis of specific proteins in the submaxillary gland by removing or counteracting a repressor-like substance normally present in the female glands.

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