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STUDIES ON PEROXIDASE-CATALYSED FORMATION OF THYROID HORMONES ON A PROTEIN ISOLATED FROM SUBMAXILLARY GLAND

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Summary

A protein has been solubilized and purified to homogeneity from the microsomal fraction of goat submaxillary gland. This protein can preferentially be iodinated to form triidothyronine and thyroxine with the help of submaxillary peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) solubilized and purified from the same microsomal fraction. The protein can also be isolated from soluble supernatant and was found to be identical to the microsomal protein as judged by their molecular properties as well as the formation of triiodothyronine and thyroxine. The protein has the molecular weight of 120 000 and contains two unequal subunits of molecular weight of 80 000 and 44 000. The molecular weight of the peroxidase is 72 000 and consists of a single polypeptide chain. The enzyme has the R_z value of 0.4 and is inhibited by azide and cyanide. Mersalyl, a mercurial, strongly inhibits the enzyme activity while *N*-ethylmaleimide cannot. The enzyme can catalyze the formation of 62 μmol of I_3^-/min per mg of protein at its optimum pH of 5.2. The apparent K_m for H_2O_2 and KI is $0.16 \cdot 10^{-3}$ M and $1 \cdot 10^{-3}$ M, respectively.

Introduction

We reported earlier that the soluble supernatant fraction of goat submaxillary gland could catalyze the formation of monoiodo- and diiodotyrosine when tyrosine and monoiodotyrosine were used as substrates [1,2]. The peroxidase (donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7) involved in this iodination reaction was partially purified and some of its catalytic properties were described [3]. The importance of this submaxillary peroxidase on

biological iodination under thyroidectomized condition was also reported from our laboratory [4]. Taurog and Evans [5] also presented evidence for the extra-thyroidal biosynthesis of thyroxine. As thyroglobulin is the physiological substrate for thyroid hormone formation catalyzed by thyroid peroxidase, we became interested to investigate whether the submaxillary gland contains any special protein, like thyroglobulin which may act as the substrate for the formation of triidothyronine or thyroxine catalyzed by the sumaxillary peroxidase. The present communication deals with the isolation and characterization of such a protein from the submaxillary gland of goat and some aspects of triidothyronine and thyroxine formation on this protein catalyzed by the submaxillary peroxidase isolated as a bye product.

Materials and Methods

Chemicals. Sodium deoxycholate, glucose oxidase, bovine serum albumin, monoiodotyrosine, diiodotyrosine, triiodothyronine, thyroxine, DEAE-cellulose, lactoperoxidase (EC 1.11.1.7) and Coomassie brilliant blue were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). $Na^{131}I$ (carrier free) was procured from Bhaba Atomic Research Centre, Bombay, India. Sephadex G-100 and Sephadex G-200 were supplied by Pharmacia Fine Chemicals. Pronase was obtained from Serva Feinbiochemica. X-ray films (ORWO HS 11, made in G.D.R., processed by Hindustan Photo Films Manufacturing Co. Ltd. India) were purchased from HPEM Co. Ltd., India. Other reagents were of analytical grade.

Collection of gland. The submaxillary glands of goat were collected on ice from the local slaughter house and stored at −20°C before use. Usually fresh glands were used but frozen glands were stable for a month,

Subcellular fractionation. The glands (40 g) were washed to remove blood, defatted as far as possible and homogenized in 20 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose (SP buffer) to get 10% homogenate. The homogenate was centrifuged at 700 × *g* for 10 min to remove cell debris and nuclear fraction. The supernatant was spun at 8000 × *g* in a Sorvall RC-2B refrigerated centrifuge for 15 min to obtain mitochondrial fraction. The post-mitochondrial fraction was centrifuged at 105 000 × *g* for 1 h to get microsomal fraction and soluble supernatant. The mitochondrial or microsomal fraction was suspended to a protein concentration of 20 mg/ml. All these steps were carried out between 0 and 4°C.

Assay of peroxidase activity. The peroxidase activity was measured according to Alexander [6] by measuring the increase in the absorbance of I_3^- formed due to oxidation of KI at 353 nm in a Zeiss PMQ II spectrophotometer. The reaction mixture contained 1 mM KI, 100 mM acetate buffer, pH 5.24, 0.16 mM H_2O_2 and a suitable amount of the fractions or purified peroxidase. Activity was expressed as the change in absorbance at 353 nm/min per mg of the protein. Amount of I_3^- formed was calculated from the molar extinction coefficient of I_3^- of 22 600 [6].

Iodination of protein. Iodination of microsomal protein was carried out by incubating it at 37°C for 10 min with the following reagents in a final volume of 1 ml : 0.4 mM KI containing 200 000 cpm of $Na^{131}I$, 100 mM acetate

buffer, pH 6.0, 0.2 mM H_2O_2 (or 40 μg glucose oxidase and 10 mM glucose) and 0.5 mg of microsomal protein. The reaction was initiated by the addition of H_2O_2 or glucose oxidase. For microsomal protein iodination, the fraction itself acted as the source of peroxidase.

In case of purified protein, iodination was carried out exactly as mentioned above except that the system was supplemented with purified submaxillary peroxidase (6—8 μg) or lactoperoxidase (2—4 μg) and the incubation was extended up to 120 min. Although iodide incorporation into the protein was found to be linear for 10 min, the amout of triiodothyronine and thyroxine formed by coupling reaction was found to be significantly higher at 120 min. However, as the rate of formation of triiodothyronine was not linear with time, the result was, therefore, expressed as nmol of product formed/120 min per mg of protein. The reaction was stopped by the addition of 1 mM sodium thiosulphate. Unreacted iodide could be removed by (i) passing the whole reaction mixture through an ion-exchange resin column (Dowex Cl^-), or (ii) precipitating the protein with trichloroacetic acid. Radioactivity was measured in a γ-ray solid scintillation counter.

Pronase digestion, extraction and autoradiography. The iodinated protein(s) was suspended in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, and digested with pronase (protein:pronase = 1:0.25 w/w) for 8 h at 37°C. The product obtained after digestion was extracted with 2 ml of *n*-butanol and repeated once. The combined concentrated butanol extract as well as the standards (triiodothyronine, thyroxine and ^{131}I) were spotted on Whatmann No. 3 chromatographic paper and was routinely run in an ascending system for 16 h in butanol/C_2H_5OH/2 N NH_4OH in a ratio of 5 : 1 : 2. However, for proper identification of the iodinated products, two-dimensional paper chromatographic separation was employed using *tert*-amyl alcohol/NH_3 (3 : 7) in a descending order (run for 24 h) and then in second direction in butanol/7 N NH_3 (1 : 1) in ascending order (run for 9 h). After drying, autoradiography was carried out by placing the paper in contact with the X-ray film for 24 h. The standards were detected according to Gmelin and Virtanen [7]. The products obtained were counted in a γ-ray spectrometer.

Solubilization and purification. The microsomal fraction (40 mg protein in 2 ml) was treated with sodium deoxycholate (final concentration 1%, w/v), incubated at 37°C for 30 min and kept frozen at −20°C. It was thawed, diluted to 10 ml with SP buffer and centrifuged at 105 000 × *g* for 1 h. 50% of the total protein was solubilized by this procedure with a recovery of 70—80% of the total counts when microsomes were iodinated prior to solubilization.

Sephadex G-100 column chromatography. The solubilized preparations was concentrated in carbowax 6000 and passed through a Sephadex G-100 column (70 cm × 1.2 cm) equilibrated with 20 mM potassium phosphate buffer containing 0.25% sodium deoxycholate. Elution (2 ml fraction) was carried out with the equilibrating buffer at a flow rate of 0.5 ml/min. The protein was resolved into two peaks. A similar elution profile was obtained with solubilized iodinated microsomal protein under identical condition. About 80% of the total counts applied on the column was recovered in the first peak.

DEAE-cellulose column chromatography. The pooled peak I from Sephadex G-100 column was concentrated to half of the original volume in carbowax

6000 and kept frozen overnight. After thawing and dialysing overnight against 500 ml of 10 mM acetate buffer, pH 5.7, the dialysed material was centrifuged at 10 000 × *g* for 10 min. The supernatant obtained was found to be free from any peroxidase activity while the slimy preparation obtained at the bottom of the tube contained all the peroxidase activity. These steps were necessary to separate peroxidase from other proteins. The supernatant was dialysed overnight against 20 mM Tris-HCl buffer, pH 8.0, and applied on DEAE-cellulose column (6 cm × 0.6 cm) previously equilibrated with the dialysing buffer. Elution was performed batchwise with increasing concentration of the same buffer. A typical elution profile is shown in Fig. 1. The protein eluated at 200 mM eluting buffer was found to be a single protein and produced significant amounts of triiodothyronine and thyroxine on iodination with purified submaxillary peroxidase. When the concentrated iodinated fraction from Sephadex G-100 column was processed in a similar fashion, 60—65% of the counts were eluted at 200 mM elution as a single protein as judged by polyacrylamide gel electrophoresis and molecular permeation chromatography on Sephadex G-200 . Other peaks eluted on washing and at higher concentration of buffer contained rest of the counts.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis without sodium dodecyl sulphate (SDS) was carried out according to Davis [8]. The 7.5% gel was run for 3 h using 3 mA of current per tube in a buffer of Tris/glycine, pH 8.3. The gels were fixed in 10% trichloroacetic acid, stained overnight with Coomassie blue (0.25% in 20% CH_3OH and 7.5% acetic acid) and destained in destaining solution (5% CH_3OH and 7.5% acetic acid). SDS-polyacrylamide gel electrophoresis was done according to Weber and Osborn [9].

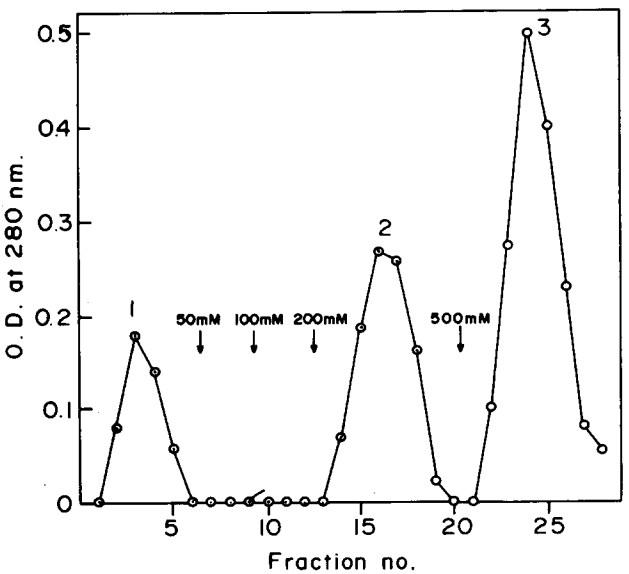

Fig. 1. DEAE-cellulose chromatography of the pooled fractions of the first peak obtained from Sephadex G-100 column. 5.0 mg of the protein was dialysed against equilibrating buffer and applied on the column. After initial washing with the equilibrating buffer batchwise elution was performed as indicated by the arrow.

The 7.5% gel was run for 8 h using 6 mA of current per tube. The fixing, staining and destaining procedures were the same as discussed above.

Molecular weight determination. Molecular weight of the native protein was determined by the method of Andrews [10]. Sephadex G-200 was suspended in 10 mM KCl at room temperature for 6 days. The column (70 cm × 1.2 cm) was set up and equilibrated with 50 mM Tris-HCl, pH 7.5, containing 100 mM KCl. The standard proteins used were bovine serum albumin (67 000), yeast alcohol dehydrogenase (130 000), catalase (260 000) and urease (460 000).

Protein estimation. The protein was estimated by following the method of Lowry et al. [11] using bovine serum albumin as standard.

Results

Distribution of iodinating activity and iodinable protein in different subcellular fractions

It was reported from our laboratory that iodinating enzyme was present in mitochondrial, microsomal and soluble supernatant fractions [1—3]. However, the specific activity of the enzyme was maximum in microsomal fraction. Our results also indicated that microsomal fraction contained maximum iodinating activity as well as the protein which could be readily iodinated with highest specific activity. However, soluble supernatant and mitochondrial fraction also showed both the activities. The specific activities of both iodinating enzyme and iodinable protein were in the order of microsome > soluble supernatant > mitochondria.

Purification of the microsomal peroxidase

A typical purification procedure for the peroxidase was shown in Table I which indicates that the enzyme was purified to about 118-fold with a yield of about 1.5%. The table also shows that the total activity of the peroxidase was increased 2-fold in the last step of purification. This could be due to removal of any inhibitor, but addition of supernatant obtained after centrifugation of the dialysed preparations of Sephadex G-100 peak I fraction as well as the concentrated dialysate failed to restore the original activity. Hence, the possibility of inhibitor was ruled out. This increase in activity occurs only after this step of purification and come back to normal on storing. The enzyme activity was also found to be a linear function of time for the various steps of purification

TABLE I

PURIFICATION OF PEROXIDASE FROM THE MICROSOMAL PREPARATION OF GOAT SUBMAXILLARY GLAND

Fractions	Total protein (mg)	Total activity	Specific activity (activity/mg)	Purification (-fold)
Microsome	24	102	4.17	1
1% deoxycholate-solubilized preparation	14.10	90	6.38	1.52
Sephadex G-100 peak I	6.25	80.20	12.83	3.07
Slimy suspension	0.35	172	491.40	117.8

indicating that the enzyme followed the normal reaction kinetics. Hence we cannot afford any explanation at present for the observed increase in the total activity during this step of purification.

Properties of the peroxidase purified from microsomal fraction and soluble supernatant

A peroxidase from the soluble supernatant of submaxillary gland of a goat was partially purified by Mahajani et al. [3]. We have purified the microsomal peroxidase while we were trying to purify an iodinable protein from the microsomal fraction. The properties of these two peroxidases were studied as shown in Table II. The table shows that some of the catalytic properties of this enzyme isolated from supernatant and microsomal fraction are different to some extent. $K_{m(app)}$ of KI for both the peroxidases was almost the same. The difference in other properties, e.g. K_m of H_2O_2, pH optima may be due to different procedures used for peroxidase assay. Mahajani et al. [3] used both *O*-dianisidine and KI as electron donors and followed the reaction by measuring oxidation of *O*-dianisidine at 460 nM while only KI was used in our assay system and I_3^- formation was followed at 353 nM to measure enzyme activity. The peroxidase from soluble supernatant formed 31 μmol of I_3^-/min per mg of protein and our best preparation from microsomal fraction produced 62 μmol of I_3^-/min per mg of protein. As regards the formation of iodotyrosines, our preparations showed more incorporation of I^- into tyrosine or monoiodotyro-

TABLE II

PROPERTIES OF THE PEROXIDASE PURIFIED FROM MICROSOMAL FRACTION AND SOLUBLE SUPERNATANT

Soluble supernatant indicates the data reported by Mahajani et al. [3] on the peroxidase activity assayed at 460 nm using both *O*-dianisiding and KI as electron donors. Microsome indicates data obtained on the peroxidase activity assayed at 353 nm using only KI as electron donor. n.d., not determined.

Properties	Peroxidase purified from:	
	Solunle supernatant	Microsome
1. $K_{m(app)}$ for KI	$1.3 \cdot 10^{-3}$ M	$1.0 \cdot 10^{-3}$ M
2. $K_{m(app)}$ for H_2O_2	$6 \cdot 10^{-5}$ M	$16 \cdot 10^{-5}$ M
3. pH optima	4—4.5 (sodium citrate/sodium phosphate buffer)	5.2 (sodium acetate buffer)
4. μmol of I_3^- formed/min per mg protein	31	62
5. nmol of I^- incorporated/min per mg protein into:		
(a) Tyrosine	110	166
(b) Monoiodotyrosine	112	170
6. Percent inhibition with SH-blocking agent		
(a) *N*-Ethylmaleimide (5 mM)	0	0
(b) Mersalyl (1 mM)	n.d.	85
(c) Mersalyl (1 mM) + dithiothreitol (5 mM)	n.d.	0
7. R_Z value	0.4	0.4
8. Percent inhibition with:		
(a) Sodium azide (1 μM)	86	95
(b) Sodium cyanide (1 mM)	100	100
9. Molecular weight	n.d.	72 000
10. Number of polypeptide chains	n.d.	1

sine. These indicate that our preparation was more active than Mahajani's preparation. Mahajani et al. [3] did not demonstrate the presence of active -SH group of the peroxidase. Table II indicates that 1 mM mersalyl inhibited 85% of the peroxidase activity and 5 mM dithiothreitol completely reversed the inhibition. *N*-Ethylmaleimide was, however, ineffective. This suggests that the functional -SH group is present in the hydrphobic region of the enzyme molecule which was inaccessible by relatively hydrophilic *N*-ethylmaleimide. Both the peroxidases showed same R_z value and almost same type of inhibition with azide and cyanide which interact with the heme group of the enzyme. These suggest the presence of ferric protoporphyrin as prosthetic group in these two enzymes. Previous workers could not obtain homogeneous preparation and hence molecular weight and presence of subunits were not determined. However, we obtained a homogeneous preparation of the microsomal peroxidase as revealed by polyacrylamide gel electrophoresis without sodium dodecyl sulphate as indicated in Fig. 2a. No contaminating protein was detected even after applying 100 μg of the purified preparation. The lower limit of sensitivity for peptide detection under the present condition of gel electrophoresis was found to be 10 μg. When the same preparation was tested on polyacrylamide gel electrophoresis with sodium dodecyl sulphate, it showed a single polypeptide band of molecular weight of 72 000.

Purification of the iodinable protein

Table III shows the steps of purification of the iodinable protein from the microsomal preparation of the submaxillary gland of goat. After DEAE-cellulose column chromatography, the protein was purified 15-fold with a final

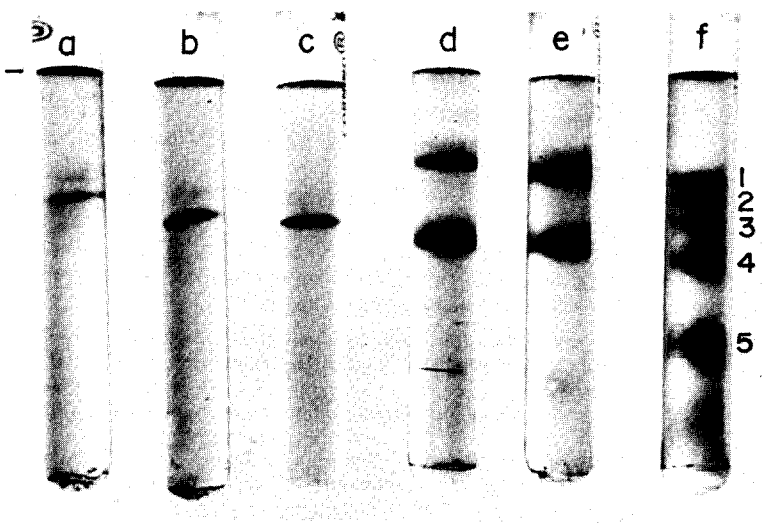

Fig. 2. Polyacrylamide gel electrophoresis of the protein(s). (a) 40 μg of purified peroxidase was used for gel electrophoresis without SDS; (b and c), gel electrophoresis of 40 μg of the purified protein from microsome or soluble supernatant without SDS; (d and e), same as (b and c) with SDS using 100 μg of protein; (f) SDS gel electrophoresis of (1) lactoperoxidase (78 000), (2) bovine serum albumin (67 000), (3) ovalbumin (43 000), (4) trypsin (24 000) and (5) cytochrome *c* (12 000) as standard proteins.

TABLE III

PURIFICATION OF THE IODINABLE PROTEIN FROM THE MICROSOMAL FRACTION OF SUBMAXILLARY GLAND OF GOAT

Steps	Total protein (mg)	Total cpm	Specific activity ($\times 10^3$) (cpm/mg protein)	Purification (-fold)	Yield (%)
Microsome	40	350 000	8.8	1	100
1% deoxycholate-solubilized preparation	19.5	245 000	11.2	1.2	50
Sephadex G-100 1st peak	5.0	170 000	34	4.0	12.5
DEAE-cellulose (2nd peak)	0.8	104 000	130	14.7	2.1

yield of 2%. On iodination of the protein in the individual peak obtained from DEAE-cellulose column (Fig. 1) and subsequent analysis of the products formed, it was found that the protein of peak 2 (200 mM eluate) showed highest incorporation of radioiodide and highest formation of triiodothyronine and thyroxine in comparison to proteins in peak 1 and peak 3 as shown in Table IV. This protein has also been found to be present in the soluble supernatant fraction of the same gland. The purification procedure from this fraction was almost the same except the elution of the protein from the DEAE-cellulose column. In this case, after washing the column with 100 mM of Tris-HCl, pH 8.0, the column was eluted with a linear gradient from 100 mM to 600 mM of Tris-HCl, pH 8.0, instead of batchwise elution. The required protein was eluted at 250—275 mM concentration of the same buffer and showed similar mobility and characteristic in non-dissociating and dissociating polyacrylamide gel electrophoresis, respectively.

Properties of the protein

Formation of triidothyronine and thyroxine: The purified protein from both the microsomal and soluble supernatant fraction produced significant amounts of triiodothyronine and thyroxine on iodination with purified submaxillary

TABLE IV

PRODUCT FORMATION BY THE PROTEINS OF DIFFERENT PEAKS FROM DEAE-CELLULOSE COLUMN AND PURIFIED PROTEIN FROM SOLUBLE SUPERNATANT

In this particular experiment 0.5 mg of protein(s) of different fractions from DEAE column or the purified soluble supernatant protein was used for iodination. The incubation was carried out for 2 h at 37°C.

Protein(s)	nmol of products formed/mg protein in 120 min incubation time				
	Total	Monoiodo-tyrosine	Diiodo-tyrosine	Triiodo-thyronine	Thyroxine
1st peak (washing)	1.296	0.80	0.36	0.066	0.07
2nd peak (200 mM eluate)	3.60	2.16	0.92	0.25	0.27
3rd peak (500 mM eluate)	1.698	0.98	0.54	0.083	0.095
Purified protein from soluble supernatant	3.40	2.06	0.87	0.22	0.25

peroxidase. Table IV also shows (2nd peak vs. purified protein from soluble supernatant) the formation of different products on iodination of the protein isolated from the microsome or the soluble supernatant fraction. The results indicate that the amount of the different products formed are almost the same in both cases. Typical autoradiograms for identification of the products after one-dimensional and two-dimensional chromatographic separation of the products are shown in Fig. 3a and b, respectively. Iodination of the purified protein was carried out at different pH from 4.5 to 7.0 and the optimum pH for maximum iodination as well as the triiodothyronine and thyroxine formation was found to be 6.0 in both glucose-glucose oxidase and direct H_2O_2-added systems. However, glucose-glucose oxidase as H_2O_2-generating system was found to be more effective than direct addition of H_2O_2. The iodination of the protein molecule was found to be linear up to about 10 min at 37°C and up to the protein concentration of about 100 μg. However, the coupling reaction for the formation of triiodothyronine and thyroxine was found to occur slowly beyond that time (10 min) at least up to 2 h although the rate of formation was not found to be linear with time during this period of time. The optimum concentrations for KI and H_2O_2 were found to be 0.4 mM and 0.2 mM, respectively, using glucose oxidase and submaxillary peroxidase of 15 μg and 6 μg, respectively.

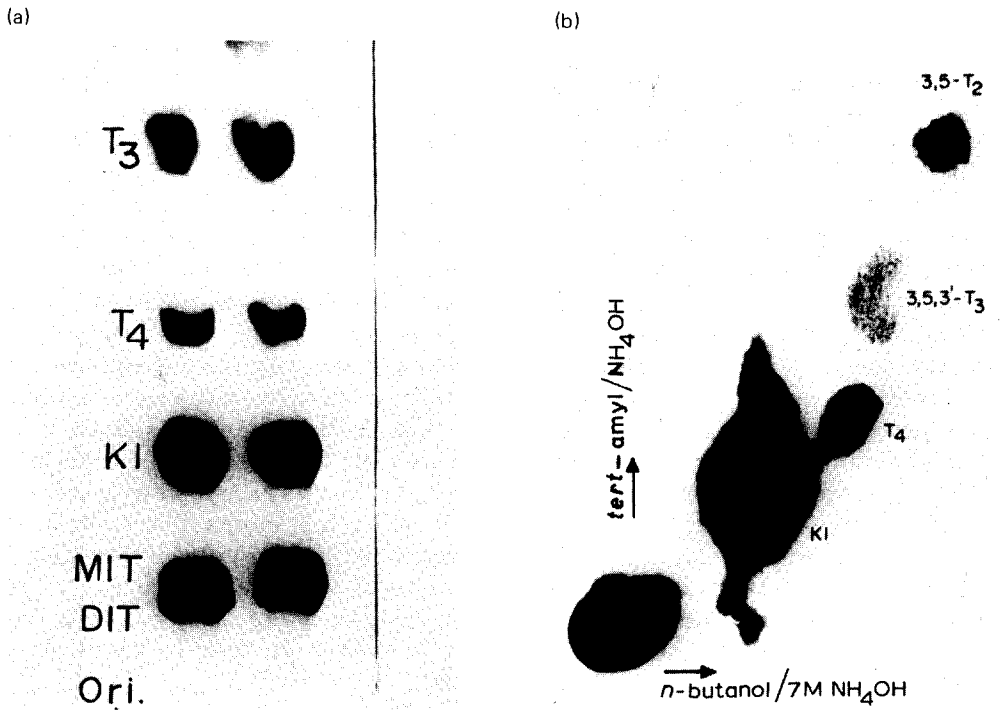

Fig. 3. Autoradiograms of the products obtained after pronase digestion of the iodinated protein. (a) One-dimensional chromatography using butanol/C_2H_5OH/2 N NH_4OH in the ratio of 5 : 1 : 2 in ascending system, and (b) two-dimensional chromatography as indicated in the figure using *tert*-amyl alcohol/NH_4OH (3 : 7) in the first direction and butanol/7 N NH_4OH (1 : 1) in the second dimension.

Physical properties

The purified protein from the microsomal as well as the soluble supernatant fractions was subjected to non-dissociating and dissociating polyacrylamide gel electrophoresis. In non-dissociating gel the protein showed a single polypeptide band as shown in Fig. 2b and c. When the same preparation was applied on SDS-polyacrylamide gel electrophoresis, it showed two distinct polypeptides corresponding to the molecular weights of 80 000 and 44 000, respectively (Fig. 2d and e) when compared with the standard proteins as shown in Fig. 2f. The molecular weight of the native protein was determined by molecular permeation chromatography in Sephadex G-200 according to Andrews [10]. The proteins, both from microsomal or soluble supernatant, were eluted with a single symmetrical peak in the same elution volume from the Sephadex G-200 column. From the calibration curve of the elution volume of the standard proteins, the molecular weight was found to be 120 000, which is very close to the additive molecular weights of the two polypeptide subunits.

Discussion

The biological significance of the salivary gland was not known for a long time except the secretion of saliva. However, several factors, like epidermoid growth factor [12], nerve growth factor [13], lymphoid tissue inhibitory factor [14] and glucagon-like substance have been recently isolated from mouse submaxillary gland [15]. The authors suggested that submaxillary gland may serve both exocrine and endocrine functions [15,16].

The role of salivary glands in biological iodination reaction is not yet well established. Taurog and Evans [5] and other authors [17,18] have suggested that the gland may act as a standby for extrathyroidal biosynthesis of thyroid hormones. Various workers have already shown a highly active iodinating peroxidase in the submaxillary gland [1—4]. A soluble iodinating peroxidase was isolated in our laboratory from goat submaxillary gland [3]. However, the isolation of the peroxidase described in this communication deserves special mention. Like thyroid peroxidase, this enzyme is membrane bound and requires detergent treatment for solubilization. The method of isolation is very simple, less time consuming to obtain a homogeneous enzyme of very high specific activity. This enzyme is also capable of forming significant amounts of triiodothyronine and thyroxine on the protein isolated from the same source. This protein may presumably serve as a backbone for biological iodination in the submaxillary gland. Sarimo and Tenovuo [19] and Tenuvuo and Sarimo [20] suggested that albumin and amylase may act as the acceptors of iodide in human saliva. However, the isolated protein from the submaxillary gland of goat was neigher albumin nor amylase as evidenced by the polyacrylamide gel electrophoresis with and without sodium dodecyl sulphate as well as by the assay of amylase activity. The physiological significance of this protein is not yet clearly known and is currently under investigation. Studies are now in progress of characterize this protein in details.

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