Purification and Properties of an Iodide Peroxidase from Submaxillary Gland of Goat

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An iodinating enzyme has been identified and partially purified from the homogenate of goat submaxillary gland. The iodinating enzyme was purified 130-fold and found to be closely associated with iodide peroxidase activity in every step of purification. The finally purified preparation, although not homogeneous, exhibited both iodinase and peroxidase activities. The iodinase and the iodide peroxidase activities have comparable pH curves, hydrogen peroxide requirement and similar reactions towards some common reagents. Partial inactivation by pronase digestion also affects the two activities in a similar manner and fails to dissociate them. These observations led us to suggest that a single enzyme is responsible for the oxidation of I^- and incorporation of active iodine to the tyrosine moiety. The enzyme catalyses a production of 31.0 μmol triiodide $\times min^{-1} \times mg^{-1}$ and can also catalyse effectively the oxidation of o-dianisidine which is stimulated 2000% in presence of KI. The enzyme also catalyses the iodination of thyroglobulin and albumin, other than free tyrosine, with the formation of iodotyrosines.

Peroxidation of iodide and organification of active iodine by incorporation into tyrosine moiety of thyroglobulin are the two important steps in the biosynthesis of thyroid hormones; the enzyme(s) catalysing the reactions has been well demonstrated *in vitro* [1—7]. A controversy still exists whether both the reactions are catalysed by the same enzyme or whether two different enzymes are involved for these two separate half reactions. Fawcett and Kirkwood [1] first suggested that besides peroxidase a separate tyrosine iodinase is necessary for the process and this was further extended by DeGroot and Davis [3] and also by Yip [4]. However, the concept that the same enzyme catalyses both the reactions was put forward by Alexander and Corcoran [8] and is further supported by purification of a number of thyroid peroxidases from a variety of species all of which catalyse both the reactions [6,9].

Iodide peroxidase-tyrosine iodinase has also been shown to be present in some extrathyroidal tissues, [1,2] of which salivary glands need special mention [1,2,10—12]. Morrison *et al.* [12] have isolated and purified a peroxidase from bovine submaxillary gland and further demonstrated that the enzyme is identical to lactoperoxidase. It has been reported earlier from our laboratory that the extract of goat submaxillary gland catalyses the formation of iodotyrosines [13,14] and the same preparation also exhibits a different type of peroxidase activity which is stimulated by KI [15]. Recently, a typical hemoprotein peroxidase has been purified to homogeneity from the same source [16]. Since this pure protein did not catalyse iodination reactions [16] whereas the homogenate did, attempts were made to purify the iodinating enzyme. The present communication deals with the isolation, purification and properties of an iodide-peroxidase tyrosine-iodinating enzyme with special emphasis to the similarity between the iodinating and peroxidatic activities of our enzyme preparation.

EXPERIMENTAL PROCEDURE

Materials

Submaxillary glands were collected from the local slaughter house. L-Tyrosine, monoiodotyrosine, diiodotyrosine and thiouracil were procured from Nutritional Biochemical Corporation. CM-cellulose was purchased from Bio-Rad Laboratories. Histidine, tryptophan, crystalline heme (bovine hemin), sodium borohydride and DEAE-cellulose were products of Sigma Chemical Company. Fluka AG (Chemische Fabrik, Buchs SG, Switzerland) supplied acrylamide, N,N'-methylenebisacrylamide, N,N,N',N',-tetramethylethylenediamine, o-dianisidine and bovine

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Abbreviations. CM-cellulose, carboxymethyl-cellulose; R_z, ratio of absorbance at 408 nm to that at 280 nm.

Enzyme. Peroxidase (EC 1.11.1.7).

serum albumin; Dowex 50W-X8 (ionic form Na^+, 20—50 mesh), N-ethylmaleimide, guaiacol and 2,4,6-collidine were purchased from British Drug House. L. Light and Company supplied p-chloromercuribenzoate. $Na^{131}I$ was obtained from Bhabha Atomic Research Centre (Trombay, Bombay, India). Freund's complete adjuvant and bacto agar were purchased from Difco Laboratories. Other chemicals used were of reagent grade.

METHODS

Assay of Iodinating Enzyme

The enzyme was assayed by measuring the amount of radioactive iodine incorporated into tyrosine. The reaction mixture contained in a final volume of 3.0 ml, 300 μmol sodium citrate—sodium phosphate buffer pH 4.4, 0.5 μmol L-tyrosine, 0.8 μmol potassium iodide and 15 μCi $Na^{131}I$ (carrier-free) and a suitable volume of the enzyme preparation. The reaction was started by the addition of 0.5 μmol hydrogen peroxide and was stopped exactly at the tenth minute by the addition of 0.6 ml of $50^0/_0$ trichloroacetic acid. The amount of radioactive iodide incorporated into tyrosine was assayed following the method of Alexander and Corcoran [8]. 2.0-ml aliquots of the incubation mixture were transferred immediately, to minimize non-enzymatic iodination, to Dowex 50W-X8 (Na^+ or H^+ form) columns (0.8 ×6 cm), where the organically bound iodine became adsorbed. The columns were washed four times with 10 ml distilled water to get rid of unreacted inorganic iodide. The organically bound radioactive iodocompounds, remaining on the column after washing, were measured by holding the columns in a fixed geometry in a solid scintillation counter attached to a γ-ray spectrometer (Model-GRS 20B, Electronic Corporation of India Limited).

For the determination of exact amount of monoiodotyrosine and diiodotyrosine formed, the chromatographic and radioautographic method of Hati and Datta [14] was followed.

A unit of iodinating activity is defined as the amount of enzyme required to incorporate 1 μmol iodine into tyrosine under the present assay conditions.

Assay of the Iodide-Peroxidase

The enzyme activity was measured as the increase in the absorbance at 460 nm in a Carl Zeiss spectrophotometer, Model P.M. Q.II, using 1.0-cm light path. Except for the addition of KI, the method was essentially the same as that followed by Yip [17]. The reaction mixture contained in a final volume of 3.0 ml, 200 μmol sodium citrate—sodium phosphate buffer pH 4.4, 2.0 μmol KI, 0.5 μmol o-dianisidine in 0.05 ml methanol and 0.01 to 0.05 ml of the

enzyme preparation in suitable dilution. 0.2 μmol hydrogen peroxide was added last to start the reaction, mixed well and readings were taken at 5-s intervals for 30 s. o-Dianisidine solution in methanol was prepared fresh before use.

A unit of enzyme is defined as the amount of enzyme which causes an absorbance change of one unit per minute at 460 nm under the assay conditions described.

Triiodide (I_3^-) formation was assayed under essentially similar conditions, only o-dianisidine was omitted from the assay system and 4.0 μmol KI was added instead of 2 μmol and the absorbance change was followed at 353 nm, nmol I_3^- produced was calculated from the absorption coefficient of I_3^- at 353 nm [18].

Measurement of Protein

The protein content was determined by the absorbance at 280 nm [19], using crystalline bovine serum albumin as standard under identical conditions.

Polyacrylamide-Gel Electrophoresis

Disc-gel electrophoresis was carried out by the method of Davis [20]. A $7.5^0/_0$ gel, buffered at pH 8.3 with 0.2 M Tris-glycine, was used and electrophoresis was continued for 1.5 h at 0—4 °C. The gels were stained for protein with amidoschwarz and the twin gels were dipped in the reaction mixture for peroxidase assay when the region of the gel containing the enzyme was stained brown due to the oxidation of o-dianisidine.

Preparation of Antibody

Antibody against crude and purified enzyme preparation was prepared by injecting 10 mg and 1 mg of enzyme preparation from step 4 and step 6 respectively suspended in equal volume of complete Freund's adjuvant, to healthy male rabbits, thrice, at weekly intervals. Blood was drawn a week after the last injection, serum separated and used as antibody preparation. Double-diffusion plate test of Ouchterlony was carried out as described earlier [21].

Enzyme Preparation

Submaxillary glands of goat were brought to the laboratory packed in ice and stored in the deep freeze at —20 °C. The frozen glands could be used within one month.

Preparation of Subcellular Fractions. Unless otherwise stated, all steps of enzyme purification were carried out at 0—4 °C. 10 g of submaxillary glands were thawed, washed once with water to remove blood as far as possible, minced well and homogenised

in 40 ml 0.05 M sodium phosphate buffer pH 6.0, for 2 min altogether in a Waring blender (Bajaj, senior mixer, 13000 rev./min); the homogenisation was interrupted every 30 s for rechilling the suspension. The homogenate was centrifuged at $1000 \times g$ in Sorvall RC 2-B refrigerated centrifuge for 10 min. The $1000 \times g$ supernatant was termed as the crude homogenate and was taken as the starting material. This was subjected to a centrifugal force of $10000 \times g$ for 20 min. The $10000 \times g$ pellet was suspended in 10 ml same buffer, whereas the supernatant was further centrifuged at $105000 \times g$ for 1 h in a Spinco Model L preparative ultracentrifuge. The $105000 \times g$ pellet was suspended in 6.0 ml water and the supernatant referred to as the soluble supernatant.

Step 1. For the routine preparation of purified enzyme, 120 g of submaxillary glands were thawed and washed as mentioned above and homogenised in 400 ml of the buffer to give a 30% homogenate. The $1000 \times g$ supernatant was used as the starting material for further purification. This preparation is very slimy.

Step 2. The enzyme preparation from step 1 was filtered through a layer of glass wool, centrifuged at $50000 \times g$ for 1 h and the pellet was rejected. This centrifugation was adopted for routine work as there was not much difference between the specific activity and recovery of iodinating and peroxidatic activities in $50000 \times g$ and $105000 \times g$ supernatants. The recovery of activity was always more than 100%.

Step 3. 25.8 g of solid reagent-grade ammonium sulphate was added slowly with stirring to each 100 ml of the $50000 \times g$ supernatant and centrifuged for 15 min at $12000 \times g$. The precipitate was rejected, and, to the supernatant, ammonium sulphate was added again in a proportion of 12.3 g per 100 ml. After centrifugation this second ammonium sulphate precipitate was taken for further purification. The first ammonium sulphate precipitate and the final supernatant, contained only 13% and 15% of the activity respectively and were discarded. The second active ammonium sulphate precipitate was dissolved in 50 ml distilled water by homogenisation in Potter-Elvehjem homogeniser. The pink coloured precipitate dissolved with difficulty and although clear was very slimy, probably due to the presence of mucoid material. It takes usually 3—4 h to reach this step.

Step 4. The dissolved precipitate was dialysed for 4 h against 8 litres of distilled water with 7 changes to remove ammonium sulphate. The precipitate which appeared during dialysis was clarified by centrifugation at $50000 \times g$ for 15 min. This conditions of dialysis is critical as longer dialysis tends to inactive the enzyme, whereas, ammonium sulphate concentration above 0.5 mM prevents proper adsorption on the ion-exchanger column in the next step. Dialysis against 1 mM KI or 10 μM glutathione could not stabilize the enzyme. Filtration on Sephadex G-50
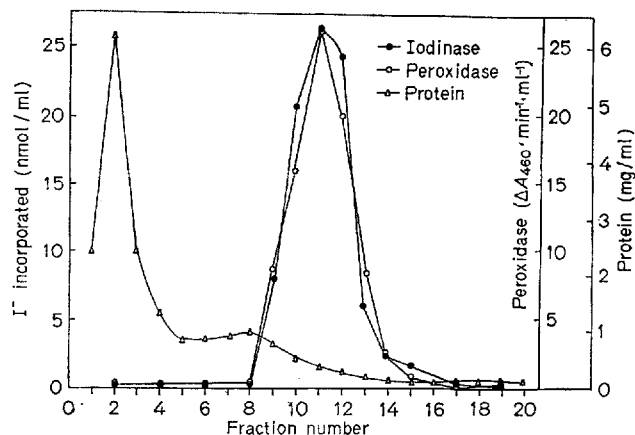

Fig. 1. *Elution profile from CM-cellulose column.* Measurement of enzyme activities and elution of enzyme has been described in the text

as an alternative method for removal of ammonium sulphate was also not successful.

Step 5. The dialysed material was then applied in batches on a CM-cellulose column (10×1.2 cm) previously equilibrated with 0.01 M sodium citrate — sodium phosphate buffer pH 5.0. Because of the slimy nature of the enzyme, it takes about 10—12 h for the enzyme to enter the resin column and get adsorbed as a concentrated red ring at the top of the column. Gradient elution was carried out with 100 ml of 0.01 M equilibrating buffer in the mixing chamber and 0.5 M of the same buffer in the reservoir; 6-ml fractions were collected with the help of a LKB automatic fraction collector. A typical elution profile is shown in Fig. 1. Fractions containing enzyme of maximum specific activity were pooled and concentrated against 60% sucrose overnight. Contents of tubes containing enzyme of lower specific activity were combined and dialysed against distilled water for rechromatography in the same way to get a higher recovery. This preparation is stable between 0—4 °C for at least 7 days and retained 80% of its activity for 14 days.

Step 6. The concentrated CM-cellulose eluate was dialysed for 2 h against 2 litres of distilled water with 3 changes, centrifuged to clarify the turbidity and applied on a column of DEAE-cellulose (10 × ×1.2 cm). The DEAE-cellulose was first activated with 0.02 N NaOH in 1.0 M NaCl, washed with water until the suspension was of neutral pH and was finally equilibrated in 5 mM sodium phosphate buffer pH 7.1. After application of the CM-cellulose eluate the column was first washed with 35.0 ml of 5 mM and then with 30 ml of 0.1 M of the same buffer, collecting 3.0-ml fractions in the automatic fraction collector. A concentrated red ring was formed 2 — 3cm from the top of the column during adsorption of the
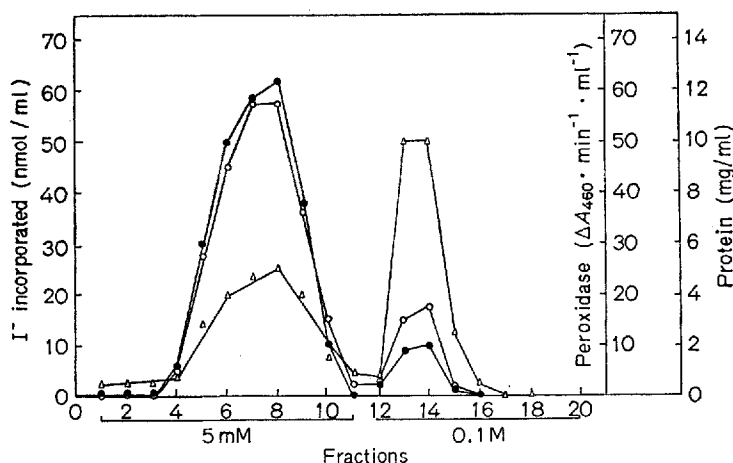
Fig.2. *Elution profile of the enzyme from DEAE-cellulose.* Enzyme activities were measured according to the procedure mentioned under Methods. Elution was carried out with 5 mM phosphate buffer pH 7.1, up to tube 11 and with 0.1 M buffer from tube 12 onwards. Volume of fractions = 3 ml. (●) Iodinase activity; (○) peroxidase activity; (△) Protein

enzyme. Fig.2 shows that two peaks were obtained, an initial colourless one associated with the present enzyme activity which was pooled, concentrated and used as the purified enzyme preparation followed by another one containing red coloured material. This latter fraction gave a sharp Soret absorption band with a maximum at 406 nm. This red coloured protein was identified as the hemeperoxidase which has recently been purified to homogeneity in our laboratory [16] from the same source. The coloured DEAE-cellulose eluate gave an R_z value of 5. This, together with its Soret peak at 406 nm indicates that it was the remaining contamination of the hemoprotein peroxidase. When the CM-cellulose eluate was subjected to electrophoresis on polyacrylamide gel at pH 8.3 and then stained for peroxidase activity as well as protein, presence of two distinct peroxidases corresponding to two distinct protein bands were revealed. The major protein band corresponded with the slow-moving peroxidase which had the same mobility as the iodide-peroxidase eluted from the DEAE-cellulose. The mobility of the faster band corresponded with the mobility of the purified hemoprotein peroxidase. It is clear therefore that the CM-cellulose eluate contained mainly iodide-peroxidase and was contaminated with the hemoprotein peroxidase while the DEAE-cellulose eluate was free from the contaminant. The characteristic spectra, R_z value, specific activity and electrophoretic mobility confirmed that the contaminant was the hemoprotein peroxidase.

The procedure outlined above was taken as the routine procedure for the purification of the enzyme and the colourless DEAE-cellulose-eluate used as the finally purified preparation.

RESULTS

Subcellular Fractionation

The crude homogenate was a thick slimy suspension containing fat, mucous and connective tissue and never gave a dependably linear assay. Therefore $1000 \times g$ supernatant was taken as the starting material for all purposes. Nearly $90^0/_0$ of the peroxidatic and iodinating activities were present in the $105000 \times g$ supernatant indicating that the enzyme(s) responsible for the two activities is soluble in nature.

Purification

The iodinase and the peroxidase were purified 132-fold and 175-fold over the $1000 \times g$ supernatant with a recovery of $7.4^0/_0$ and $9.8^0/_0$ respectively. The activities at different stages of purification are shown in Table 1. The specific activity of crude homogenate differed considerably from experiment to experiment but the degree of purification from the initial to final preparation remained the same. Fig.3 shows that several precipitin lines were formed when the antiserum produced against step-4 enzyme was tested with the same enzyme preparation. Three of the precipitin bands are distinctly visible in the figure and although two more components could be detected by careful visual inspection these were too faint to be demonstrated by photographs. CM-cellulose eluate (step 5) gave only two bands and the DEAE-cellulose eluate (step 6) gave a single precipitin line from a concentration of 10 to 200 μg. This indicates that the purified peroxidase is probably immunochemically homogeneous. However, the presence of non-antigenic impurity cannot be ruled out by this method. Polyacrylamide gel electro-

Table 1. *Purification chart of iodinase and iodide peroxidase*

| Fractions	Volume	Protein	R_z	Iodinase					Peroxidase				
				Activity	Spec. act.	Purifi-cations	Total activity	Recovery	Activity	Spec. act.	Purifi-cation	Total activity	Recovery
	ml	mg/ml		nmol/ml	$nmol \times ml^{-1} \times mg^{-1}$	-fold	U	%	U/ml	U/mg	-fold	U	%
Step 1: 1000×g supernatant	310	24	0.5	15	0.94	1	6975	100	22.5	0.63	1.0	4650	100
Step 2: 50000×g supernatant	305	9	0.4	17	2.6	2.6	7320	105	24.0	1.9	3.0	5185	112
Step 3: 2nd ammonium sulphate ppt	27	25	0.4	125	7.6	8.1	5130	70	190	5.0	8.0	3375	73
Step 4: 2nd ammonium sulphate ppt, dialysed	25	15	—	80	5.7	6.0	2850	40	114	4.0	6.3	2000	43
Step 5: CM-cellulose eluate	12×2	0.96	0.8	23	26	27.7	600	9	25	24	38	552	12
Step 6: DEAE-cellulose eluate	9	0.46	0.4	51	124	132	513	7.4	57	110	174.5	459	10
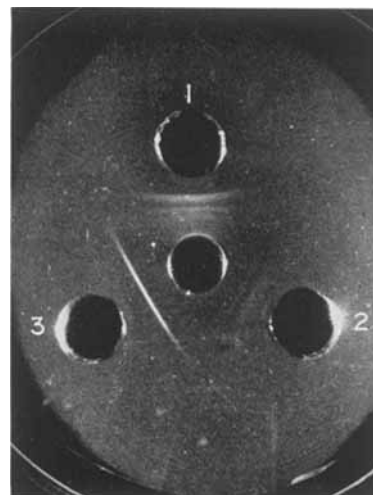
Fig. 3. *Demonstration of purity of the enzyme by immuno-diffusion.* Procedure followed was the same as described in the text. Central well, antiserum prepared against the enzyme preparation from step 4; 1, 2 and 3, enzyme preparation from step 4, 5 and 6 respectively

phoresis showed the presence of some faint contaminating protein bands. Hence, the final preparation although serologically homogeneous is still contaminated with some extraneous proteins.

PROPERTIES OF THE IODINATING ACTIVITY OF THE ENZYME

Requirements for Iodination

The various components required for iodination of tyrosine with purified enzyme are shown in Table 2. The omission of H_2O_2 or the enzyme abolished the iodination reaction indicating that the reaction was completely dependent on H_2O_2 and enzymatic in nature. When tyrosine was omitted from the assay, a predominant radioactive spot at the origin on the chromatogram was observed. This was possibly due to the iodination of tyrosine residues of the enzyme itself as suggested by Björkstén [22] whereas, in the complete system there was no spot at the origin and only dark spots in the region of monoiodotyrosine, diiodotyrosine and KI were observed.

Incorporation of radioactive iodine into tyrosine increased linearly with time of incubation up to 10 min. Increase in the activity was also proportional with increasing amount of enzyme protein up to 5 µg per 3.0 ml incubation medium, thereafter the increase in protein did not catalyse any further increase in the product formation. The results presented here are those assayed by using Dowex column, unless otherwise stated.

Table 2. *Requirements for iodinase assay*
Additions and procedure for enzyme assay are described under Methods. In this particular experiment 0.8 µg enzyme protein was used

System	I⁻ incorporated
	nmol/µg protein
Complete	112.5
Minus H_2O_2	0
Minus tyrosine	0
Minus enzyme	7.5
Complete (boiled or pronase-treated enzyme)	8.8
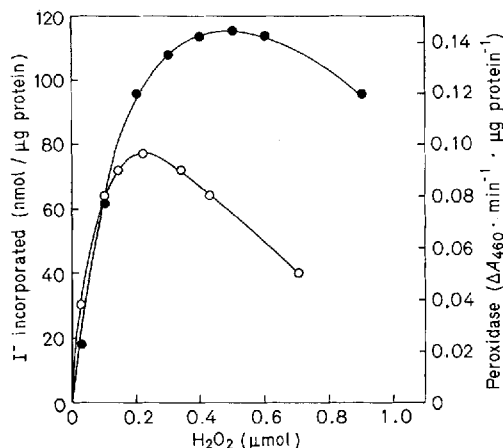
Fig.4. *Effect of varying concentrations of H_2O_2 on iodinase and peroxidase activity.* The incubation mixture and other assay conditions were same as described in the text, only the concentration of H_2O_2 was varied. 0.8 µg and 2.0 µg purified enzyme protein was used for the assay of iodinase (●) and peroxidase (O) activities respectively

Effect of Varying Concentrations of H_2O_2

Fig.4 shows that the reaction increased progressively with increasing concentration of H_2O_2 up to 0.45 µmol. Increasing the amount of H_2O_2 above 0.65 µmol caused gradual decrease of the enzyme activity. This could probably be due to the oxidation of the enzyme itself, as suggested by Weinryb [23].

pH Optimum

The enzyme showed maximum activity at pH 4.0. Alteration of pH towards the acid side caused a sharp fall in the enzymic activity, whereas no significant decrease was observed between pH 4.0—4.5 beyond which there was a gradual fall in activity (Fig.5). Although the enzyme showed slight increased activity at pH 4 than at pH 4.4, the assays were carried out at pH 4.4, the reason being discussed in connection with the experiment describing assay of iodide peroxidase at varying pH.
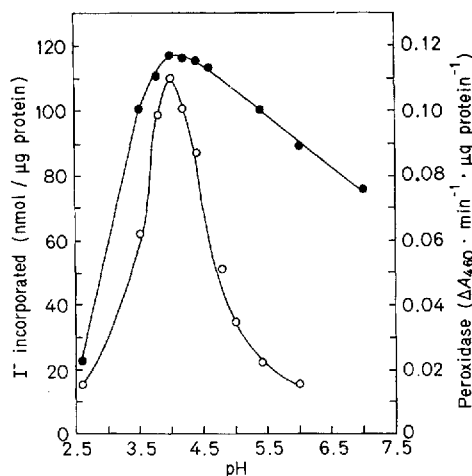

Fig.5. *pH optima of the enzyme for iodinase and peroxidase activities.* The incubation mixture and other assay conditions were same as described in the text, only the pH was varied. 0.8 µg and 2.0 µg of purified enzyme protein was used for the assay of iodinase (●) and peroxidase (O) activities respectively
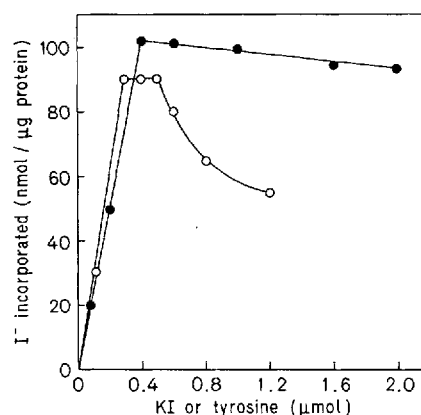

Fig.6. *Effect of varying concentrations of KI or tyrosine on iodinating activity.* Additions and experimental procedures were same as described in the text. When the effect of varying concentrations of tyrosine was studied, concentration of KI was kept fixed at 2.0 µmol, but when the effect of varying concentrations of KI was noted, 0.5 µmol tyrosine was added in the incubation mixture. 0.8 µg of purified enzyme protein was used for each assay. (●) Iodinating activity against varying concentrations of KI; (O) iodinating activity against varying concentrations of tyrosine

Effect of Different Concentration of Tyrosine and KI

The rate of reaction increased with increasing concentration of tyrosine up to 0.3 µmol per incubation volume of 3.0 ml, remained steady up to 0.5 µmol and then decreased gradually (Fig.6). When tyrosine was omitted from the incubation mixture, assays could not be performed by using Dowex column as

Table 3. *Substrate specificity*

In this particular experiment, incorporation into monoiodo-tyrosine and diiodotyrosine was followed by the chromatographic technique as described earlier and not by using Dowex column. Values are corrected for non-enzymatic reaction. In the case of tyrosine, monoiodotyrosine and diiodotyrosine, 0.5 μmol of each substrate and 0.8 μg enzyme protein was used in each incubation. In the case of albumin and thyroglobulin, 1.5 mg of each substrate and 5 μg enzyme was used. Other components of the incubation mixture were the same as described in Table 2. The reaction was stopped with 0.3 μmol thiouracil. An aliquot of the incubation mixture was digested with pronase (0.5% final concentration pH 7.6) for 17 h at 37 °C and chromatographed as usual

Substrate	I⁻ incorporated	
	Monoiodotyrosine region	Diiodotyrosine region
	nmol × min⁻¹ × μg protein⁻¹	
None	—	—
Tyrosine	88	22
Monoiodotyrosine	11	101
Diiodotyrosine	—	108
Albumin	42	145
Thyroglobulin	14	51

Fig. 7. *Exchange of ^{131}I with non-radioactive diiodotyrosine.* Incubation mixture and other assay conditions were same as described in the text. Assays were performed both by chromatographic as well as by Dowex methods. (●) Complete system; (○) boiled enzyme; (△) plus thiourea or thiouracil

the protein itself was iodinated and got adsorbed on the Dowex column giving high counts.

As shown in Fig. 6 increasing the concentration of KI up to 0.4 μmol per 3.0 ml, there was a progressive increase in the enzyme activity and thereafter it remained steady more or less up to 2.0 μmol, beyond which assays were not performed.

Substrate Specificity

The enzyme not only iodinated tyrosine but also iodinated monoiodotyrosine quite effectively (Table 3). However, it did not catalyse the iodination of some other amino acids like phenylalanine, histidine or tryptophan. When diiodotyrosine was used as the substrate, the enzyme catalysed the formation of radioactive diiodotyrosine. This is probably due to an enzyme-catalysed exchange reaction between the added ^{131}I and the iodine of diiodotyrosine. The enzyme also iodinated albumin and thyroglobulin and digestion of the incubation mixture with pronase revealed the formation of both the iodotyrosines.

Exchange of ^{131}I with Non-Radioactive Diiodotyrosine

To follow the exchange reaction essentially a similar experiment was performed as described under Methods for iodinase assay, only diiodotyrosine was used instead of tyrosine and the product was analysed chromatographically as well as by using Dowex column. Monoiodotyrosine could not be used in this experiment as the enzyme catalysed
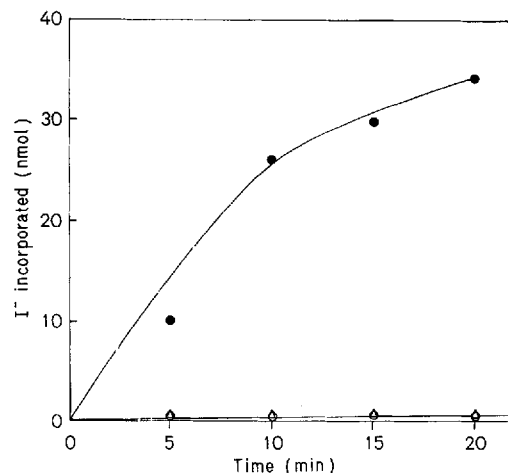
the formation of radioactive diiodotyrosine at a much faster rate than it catalyzed the exchange of added radioactive iodine with the iodine of monoiodotyrosine. Fig. 7 shows the rate of enzyme-catalysed exchange of added ^{131}I with the iodine of diiodotyrosine. The reaction was linear up to 10 min and levelled off thereafter. Absence of any exchange with boiled enzyme proves that the exchange was not a non-enzymatic one. Antithyroid agents like thiourea and thiouracil inhibited the reaction completely at a concentration of 50 μM.

Products of Iodination Reactions

It is also evident from Table 3 that when tyrosine was used as the substrate out of the total iodotyrosine formed the product was 80% monoiodotyrosine and 20% diiodotyrosine. However, if the time of incubation was increased, percentage of diiodotyrosine formation also increased to a maximum of 50%. With monoiodotyrosine as substrate, out of total radioactive product formed, 90% was diiodotyrosine and 6% was radioactive monoiodotyrosine. However when diiodotyrosine was used as substrate, thyroxine or triiodothyronine formation could not be observed; on the other hand, significant amount of radioactive diiodotyrosine was formed from nonradioactive diiodotyrosine. These results further confirm our previous finding of an exchange reaction [24].

Fig. 8 shows that the enzyme catalysed the incorporation of I⁻ into monoiodotyrosine or tyrosine with equal efficiency. In the different steps of purification also monoiodotyrosine was as effective as tyrosine
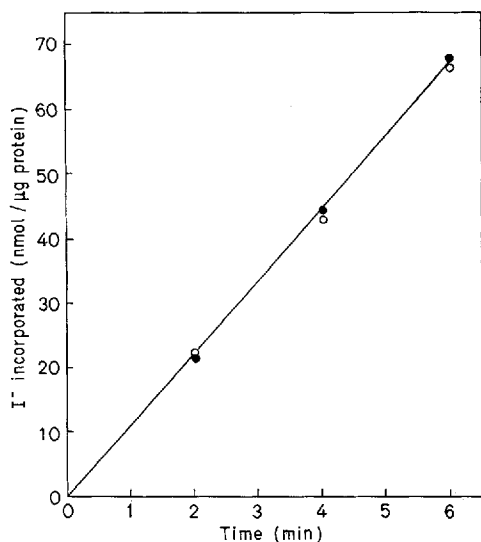
Fig. 8. *Linearity of iodinating activity against time with different substrates.* Assay conditions were the same as described earlier only either tyrosine (●) or monoiodotyrosine (O) was used as the substrate. 0.8 µg of purified enzyme protein was used for each assay
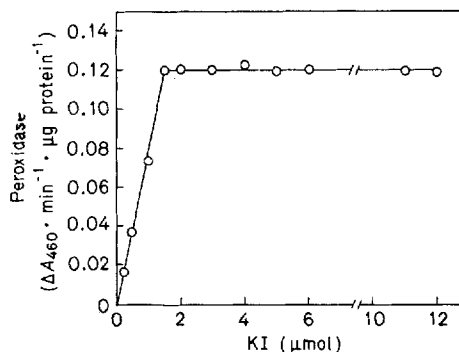

Fig. 9. *Effect of varying concentrations of KI on peroxidase activity.* The incubation mixture and other assay conditions were same as described in the text, only the concentration of KI was varied. 2.0 µg of purified enzyme protein was used for each assay

Table 4. *Requirements of iodide-peroxidase*
The procedure of peroxidase assay and additions in the incubation mixture are given under Methods. In this experiment 2 µg of enzyme protein was used

System	Peroxidase activity
	$\Delta A_{460} \times min^{-1} \times \mu g$ protein^{-1}
Complete	0.108
Minus KI[a]	0.054
Minus H_2O_2	0.00
Minus enzyme	0.00
(Boiled or pronase treated enzyme)	0.00

[a] 20 µg protein was used in this particular assay.

as a substrate. Hence the preliminary properties of the enzyme were also studied using monoiodotyrosine as the substrate.

PROPERTIES OF THE IODIDE PEROXIDASE ACTIVITY OF THE ENZYME

The purified enzyme at concentrations in the range generally used for iodination reaction effectively catalysed the peroxidation of classical peroxidase donor. This agrees well with the results of previous workers [17,25]. Hence properties of this enzyme in relation to the peroxidatic activity were studied.

Requirements for Peroxidatic Activity

Table 4 demonstrates the basic requirements of the peroxidase assay. Omission of H_2O_2 abolished the activity. With boiled or pronase-treated enzyme the activity was also nil.

Effect of KI

Yip [17] has observed that the activity of the peroxidase purified from beef thyroid was inhibited by iodide. However, as shown in Table 4, the peroxidase activity of our enzyme was stimulated $2000^o/_0$ in the presence of added KI, the percentage of stimulation varied between $1800-2200^o/_0$ from preparation to preparation. The activity increased linearly with time up to 30 s both in the presence and absence of KI, when assayed with o-dianisidine. The effect of different concentrations of KI on the rate of reaction was then studied to find out the optimum concentration of KI. As shown in Fig. 9, the activity increased linearly up to a concentration of 0.5 mM KI, and thereafter remained unchanged up to 1 mM. This result was obtained when assays were performed under normal assay condition at pH 4.4, mentioned under Methods. However, there was always slightly more stimulation by KI, around its optimum concentration, when the assays were carried out at pH 4.0 than at pH 4.4. That the enzyme catalysed the formation of I_3^- was revealed by the increase in absorbance at 353 nm. A linear relationship was observed between the enzyme activity and concentration of the enzyme protein and considering the molar absorption coefficient of I_3^- at 353 nm to be 22900 $M^{-1} \times cm^{-1}$ [18], the enzyme was found to catalyse the formation of 31.0 µmol I_3^- $\times min^{-1} \times mg$ enzyme protein^{-1}.

Table 5. *Effect of various agents on iodinating and peroxidatic activity*

Inhibitor	Concen-tration	Inhibition of	
		Iodinating activity	Peroxidatic activity
	μM	%	%
Thiourea	10	95	45
	100	100	81
Thiouracil	10	55	56
	100	100	87
Sodium cyanide	100	45	85
	1000	90	100
Sodium fluoride	100	92	70
	1000	95	100
Sodium azide	0.1	72	50
	1	93	86
Ascorbic acid	10	17	22
	100	93	100
Glutathione (reduced)	10	37	79
	100	100	100

pH Optimum

Iodide peroxidase activity of the enzyme showed a sharp optimum at pH 4.0, in sodium citrate—sodium phosphate buffer (Fig.5). It needs to be mentioned here again that the assays were routinely carried out at pH 4.4 although the enzyme activity at this pH was 18% less than that obtained at pH 4.0. This pH was preferred as the activity showed higher stability at this pH against varying concentration of KI. This discrepancy was not due to instability of the enzyme at that pH, as the enzyme was found to be stable at room temperature for at least 40 min at pH 4.0 in the presence or absence of KI.

Effect of Varying Concentrations of H_2O_2

Fig.4 shows that the maximum peroxidase activity was obtained between 60 and 100 μM. The figure also shows significant inhibition of the enzyme at higher concentrations.

EFFECT OF VARIOUS AGENTS
ON IODINATING AND PEROXIDATIC ACTIVITY

Table 5 shows the results of experiments in which various compounds were tested for possible inhibitory effects on the peroxidatic and iodinating activities of the purified enzyme preparation.

Heme Inhibitors

Cyanide was markedly inhibitory at a concentration as high as 1 mM while azide showed significant inhibition even at 0.1 μM. As is evident from the table, the iodide peroxidase activity was relatively

less susceptible to inhibition by azide and the situation is reversed in the case of cyanide. Fluoride inhibited the two activities more or less to the same extent. The inhibition of the iodinase by these compound was of the following order:

$$N_3^- > F^- > CN^-$$
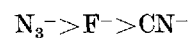
and for peroxidase the order was $N_3^- > CN^- > F^-$.

Antithyroid Agents

Antithyroid agents have been reported to be the potent inhibitors of peroxidases [17,26,27]. Both the peroxidatic as well as the iodinating activities were inhibited by thiourea and thiouracil, the latter activity being more strongly inhibited by both the inhibitors.

Ascorbic Acid and Reduced Glutathione

Both these reducing agents inhibited the activites strongly at 0.1 mM concentrations. These agents are already known to inhibit peroxidase reactions [26, 28,29]. Glutathione has earlier been proposed to be a physiological regulator of the iodination reaction in the thyroid gland [30,31]. The result obtained with the submaxillary iodinase is also compatible with this view.

Sulphydryl-Reacting Reagents

Iodoacetic acid, p-chloromercuribenzoate and N-ethylmaleimide were used as sulphydryl group attacking agents. Iodoacetic acid and N-ethylmaleimide were without any significant effect up to a concentration of 5 mM. No effect was observed even after preincubation of the enzyme with these agents. Up to 0.1 mM, p-chloromercuribenzoate had no effect and above this concentration, the reagent showed a tendency to get precipitated in the acidic pH of the incubation mixture. These observations suggest the absence of any functional —SH group in the enzyme.

Pronase Digestion
of the Enzyme Preparation

An attempt was made to digest the enzyme partially and note whether the two enzymic activities could be dissociated one from the other by pronase treatment. With this idea the enzyme was preincubated with a low concentration of pronase and aliquots were withdrawn at different intervals of time for the assay of iodinase and iodide-peroxidase activity. The results indicate that this technique could not dissociate the two activities as iodinase and peroxidase were inhibited by 50% and 48% in the first 15 min and 80% and 65% in 25 min respectively. The comparable inhibition of both the activities by pronase further suggest the hypothesis that both iodinase and peroxidase activities reside on the same

protein molecule indicating that probably the o-dianisidine oxidation site and iodide binding site is the same.

DISCUSSION

A large number of peroxidases have been reported to catalyse iodination of thyroglobulin or free tyrosine along with the peroxidation of classical peroxidase donors [6,26]. The present paper also reports similar findings with a peroxidase isolated from the submaxillary gland of goat. The enzyme was associated with the $105000 \times g$ supernatant, which is contrary to the observations made by previous workers with other peroxidases [6,17,26,27,29, 32—34]. The existence of soluble peroxidases, catalysing the iodination reaction, have been reported by Fawcett and Kirkwood [1], Morrison et al. [12] and also from our laboratory earlier [5,35]. A similar enzyme, lactoperoxidase, has been purified by Morrisson et al. [12] which also catalyses iodination of tyrosine and monoiodotyrosine. Although this enzyme catalyses the iodination reaction it shows a differential rate of iodination of tyrosine and any other derivative of tyrosine [36]. Our enzyme catalyses iodination effectively but there is no difference between the rate of iodination of tyrosine and monoiodotyrosine (cf. Fig. 8).

Very few workers have purified the iodinating enzyme, the major difficulty being encountered by the laborious and time-consuming chromatographic and radioautographic assay procedure. Spectrophotometric assay of diiodotyrosine formation [37] could not be used in the present study as monoiodotyrosine and tyrosine interferred with the assay. But by the assay procedure of Alexander and Corcoran [8], which was finally adopted during the present work, more than 30 assays could be done within an hour. The only disadvantage of this method is that the precise amounts of the different labelled compounds, e.g. monoiodotyrosine or diiodotyrosine, could not be identified. For such experiments a chromatographic method was used as described earlier [14]. Elution of the iodinated products from the Dowex column, by NH_4OH and subsequent separation by paper chromatography also gave similar results. The method not being commonly used, it was standardized with special care and the method gave us exactly parallel results as was obtained by the chromatographic method.

One may wonder why H_2O_2 has been directly added in the incubation system instead of an H_2O_2-generating system. Reports stating that slow addition of H_2O_2 can replace on H_2O_2-generating system are available [38,39]. Some workers have also used direct addition of H_2O_2 for specific experiments [17,26,28]. The only probable disadvantage of direct addition could be that H_2O_2 may inactivate the

enzyme. However, in this case a small excess of H_2O_2 did not inhibit the enzyme. The use of glucose plus glucose oxidase was avoided as inclusion of an added enzyme may cause complications in the interpretation of results and the presence of ammonium sulphate or other ions may interfere with H_2O_2 generations. Copper has been used earlier to generate H_2O_2 for iodination reaction [13—15], but in the case of the present enzyme, the incorporation was much lower and there was an initial lag period.

The ability of submaxillary gland extract to catalyse iodination reactions at a rate twice that of the thyroid gland extract, has been reported earlier [1]. In spite of the presence of thyroid gland the major function of which is thyroxine synthesis, the presence of iodinating enzyme in the submaxillary gland is fascinating. It may be mentioned here that bovine lactoperoxidase from the salivary glands has been reported to be involved in the nonthyroidal metabolism of iodide. Lactoperoxidase may also have a controlling effect on the microbiological growth in the mouth [40].

Iodination of tyrosine consists of the two following half reactions: (a) peroxidation of KI to an active form, presumably iodinium ion, and (b) incorporation of the active iodine into tyrosine. There is still a controversy whether both these reactions are catalysed by a single enzyme or two different enzymes execute the two reactions. In order to clarify the above two discrepancies, the iodinating activity along with the iodide peroxidase activity was measured simultaneously in all the steps of purification. Results reported in the present communication indicates a striking parallelism between the iodinating and iodide peroxidase activities in different subcellular fractions as well as different steps of purification. Moreover both the activities behaved identically when a few unsuccessful steps were employed to further purify the enzyme, such as precipitation with acetone or alcohol, heating the enzyme to different temperatures, filtration through Sephadex gel etc. This led us to compare the properties of the two catalytic activities of the enzyme. Similarity of pH optima, ratio of specific activities at various stages of purification, requirement of optimum concentration of H_2O_2 and KI and comparable loss of activities on pronase digestion or treatment with various inhibitors strongly suggest that both iodinase and peroxidase activities reside on the same enzyme. It may be mentioned in this connection that contrary to our observation, treatment of a partially purified thyroid peroxidase with trypsin causes a marked loss in the ability of the enzyme to catalyse iodination while the ability of the enzyme to catalyse guaiacol oxidation is unaffected [7]. These authors suggest that the two catalytic functions may involve two different active sites and although both appear to involve the heme prosthetic group the iodide binding

site is not identical to the guaiacol oxidation site. This clearly indicates that our enzyme preparation from the submaxillary gland is distinct from the enzyme in thyroid gland.

It has been mentioned in the results that our enzyme preparation is stimulated 2000% by KI. At the concentration of hydrogen peroxide used in our assays the non-enzymatic oxidation of o-dianisidine in the presence of KI was not detectable. Again, it may be mentioned that in the absence of o-dianisidine, a significant amount of free iodine was found as indicated by the appearance of blue colour when starch was added in the reaction mixture. I_2 has an absorption maximum at 460 nm [41] and may interfere with the absorbance changes measured at the same wavelength for the formation of oxidised o-dianisidine. However, a control experiment with all the components of the incubation mixture including enzyme but without o-dianisidine did not show any change in absorbance at 460 nm with time. Thus, the stimulation observed was essentially due to the increased rate of oxidation of o-dianisidine in presence of KI.

To explain how our enzyme preparation could be stimulated by KI, we propose the following scheme. In the absence of KI the normal rate of oxidation of o-dianisidine is measured by following the absorbance at 460 nm indicating the formation of oxidised o-dianisidine. However, in presence of KI, it is assumed that the iodinium ions generated by the oxidation of KI, I_3^-, I_2 or any other oxidation product of iodide may attract electrons directly from o-dianisidine and apparently demonstrate a stimulation of enzyme activity. Hence in absence of o-dianisidine only the upper part of the reaction may take place giving an increase of absorbance at 353 nm. I_3^- remains in equilibrium with $I_2 + I^-$ and hence can produce a blue colour in presence of starch which may be measured by an increase in absorbance at 612 nm.

This hypothesis is further supported by the fact that KI does not stimulate the peroxidation reaction catalysed by the hemoprotein peroxidase isolated and purified previously from the same source [16], whereas it does stimulate the same reaction catalysed

by both iodide peroxidase and lactoperoxidase. The hemoprotein submaxillary enzyme is a non-iodinating peroxidase [16] and does not catalyse the formation of I_3^- or I_2 from KI and hence there is no stimulation. Again, if an aquous solution of I_2 is added to a cuvette containing only a solution of o-dianisidine there is an increase in absorbance at 460 nm which is proportional to the amount of I_2 added. Hence, it appears that any iodide peroxidase having the capacity to further oxidise KI can be distinguished from a non-iodinating peroxidase by this method. Again addition of KI along with o-dianisidine to the incubation mixture can increase the sensitivity of the assay about 20 times.

Stimulation of peroxidatic activity by KI.

Scheme

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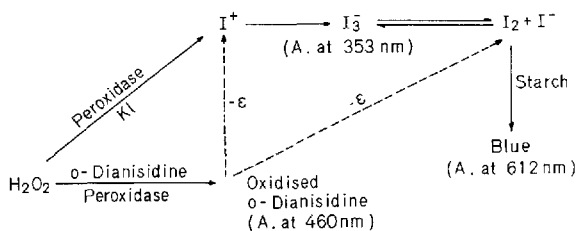
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