Biochimica et Biophysica Acta, 662 (1981) 246–255
Elsevier/North-Holland Biomedical Press

BBA 69417

PORCINE THYROID FUCOSIDASE

DEBORAH SHUEY GROVE * and GEORGE S. SERIF **

Department of Biochemistry, The Ohio State University, Columbus, OH 43210 (U.S.A.)

(Received April 9th, 1981)

Key words: Fucosidase; Thyroglobulin

An α-L-fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51) has been isolated from porcine thyroid tissue and purified 10800-fold using a combination of ion exchange, affinity and molecular sieve chromatography. The enzyme appears homogeneous by SDS electrophoresis but isoelectric focusing procedures detect considerable heterogeneity. The enzyme is a glycoprotein and this fact interferes with accurate molecular weight estimates by SDS electrophoresis or molecular sieve techniques. The enzyme appears, however, to be a tetramer and density gradient measurements set its molecular weight at 192000 ± 3000. The enzyme exhibits an optimum at a pH of 5.1 and shows a high order of specificity for L-fucose units linked through α bonds. Both sulfhydryl and carboxyl groups appear necessary for enzyme activity. The enzyme does not attack intact thyroglobulin directly but will remove fucosyl residues from the glycone moiety if the protein portion is largely removed. The enzyme thus functions in a salvage role as thyroglobulin is degraded.

Introduction

The glycone moiety of the glycoprotein thyroglobulin has been defined by the Spiro group [1] and others [2,3] and shown to contain the deoxy sugar L-fucose. Since thyroglobulin is the major protein product of the thyroid gland and a highly specialized cellular economy is directed to the synthesis of large quantities of this protein, this tissue is a convenient one in which to study L-fucose metabolism. In this regard our laboratory has investigated the action of the thyroid in utilizing preformed L-fucose and has detected and purified a L-fucokinase which converts L-fucose to β-L-fucose 1-phosphate. We have demonstrated that this enzyme is under hormonal and metabolic control [4]. In this companion study we examine the mechanism by which free L-fucose is formed in thyroid tissue, i.e., by degradative cleavage of such L-fucose-containing products as thyroglobulin by α-L-fucosidase (α-L-fucoside fucohydrolase, EC 2.7.1.52). Previous work on fucosidases has been directed to the examination of fucosidase in other tissues since a genetic disease, fucosidosis, results from its absence in humans [6]. To date however, no studies of this enzyme have been conducted with thyroid tissue.

Materials and Methods

Reagents

p-Nitrophenyl glycosides (α-D-glucoside, β-D-galactoside α-L-arabinoside, β-D-*N*-acetylglucosaminide, β-D-*N*-acetylgalactosaminide, α-D-mannoside, α-D-galactoside, β-D-fucoside, α-L-fucoside, β-L-fucoside, β-D-fucoside), 4-methylumbelliferone, L-fucose, Mops, ovalbumin (grade V), bovine α-chymotrypsinogen A (type II), bovine hemoglobin, bovine carbonic anhydrase, phosphorylase *a* from rabbit

* Present address: Fox Chase Cancer Research Institute, Philadelphia, PA 19111, U.S.A.
** To whom reprint requests should be addressed.

Abbreviations: CMC, 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulfonate; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; Temed, tetramethyl-ethylene diamine.

muscle, neuraminidase from *Clostridium perfringens*, porcine thyroglobulin (type V), horse spleen apoferritin (grade A) and *N*-ethylmaleimide were obtained from the Sigma Chemical Co. Bovine liver catalase was obtained from Worthington Biochemical Corporation. The 4-methylumbelliferyl-α-L-fucoside was obtained from Koch-Light of Colnbrook, U.K. Sepharose 6-B was obtained from Pharmacia. Agarose-ε-amino-*N*-caproyl-β-L-fucosamine was obtained from Miles-Yeda. Coomassie brillant blue R-250, Cellex D and acrylamide gel components were purchased from Bio-Rad Laboratory, Inc. The water soluble carbodiimide, 1 cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulphonate (CMC) was obtained from Aldrich Chemical Co. Porcine kidney D-amino acid oxidase and rabbit muscle lactic dehydrogenase were obtained from Boehringer, Mannheim. Oligosaccharide V, labeled with tritium at the 6th carbon of galactose, was a gift from Dr. Don Carlson of Purdue University. Unlabeled oligosaccharide V was a gift from Dr. David Aminoff of the University of Michigan. Lacto-*N*-fucopentaose I was a gift from Dr. Victor Ginsburg of NIH. Lacto-*N*-fucopentaose II was provided by Dr. Arne Lundblad of the Department of Clinical Chemistry, University Hospital, Stockholm, Sweden.

Fucosidase assays

The principal assay for the detection of α-L-fucosidase activity with synthetic substrates was a colorimetric procedure adapted from that of Opheim and Touster [6]. Activity was detected by incubating enzyme preparations at 37°C with 1.0 mM *p*-nitrophenyl-α-L-fucoside and 30 μg bovine serum albumin in 20 mM succinate buffer (pH 5.5) in a total assay volume of 150 μl. At the end of the incubation period the reaction was stopped with the addition of an equal volume of alkaline 'stop' buffer (0.133 M glycine/0.067 M NaCl/0.083 M Na_2CO_3 adjusted to a pH of 10.7 with NaOH) was pipetted into the tube. The amount of *p*-nitrophenolate anion released was then measured by absorbance (molar extinction coefficient for nitrophenol = $18.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) of the final solution at 400 nm in a Gilford spectrophotometer. 1 unit is defined as the amount of enzyme which will hydrolyze 1 μmol substrate/min at 37°C. Activities are expressed in this fashion throughout this paper.

A fluorimetric assay, based on that of Chester et al. [7] was used to determine the effectiveness of 4-methylumbelliferyl-α-L-fucoside as a substrate.

Specificity studies with oligosaccharide substrates required a different fucosidase assay since these substrates were available in restrictively small quantities and did not release a component with a high extinction coefficient or fluorescent properties upon hydrolysis. As a consequence, mucin trisaccharide, lacto-*N*-fucopentaose I, lacto-*N*-fucopentaose II and thyroglobulin were added to the assay medium described above in 0.65, 2.6, 1.9 and 1.7 mM concentrations, respectively. Incubation was carried out for 24 h at 37°C and the free L-fucose formed determined by the methods of Tsay and Dawson [8], Maler [9] or Grove and Serif [10]. The last assay is the more sensitive of these methods and permits detection of minute quantities of L-fucose (0.25–8 nmol) in the presence of proteins e.g., thyroglobulin.

Molecular weight determination

The monomer molecular weight was determined using acrylamide tube gels in a continuous phosphate system containing SDS (7.2% acrylamide, w/v), 0.19% bisacrylamide (w/v), 0.1% SDS (w/v), 0.15% Temed (v/v), 0.037% ammonium persulfate (w/v) and 0.1 M sodium phosphate buffer, pH 7.2. The running buffer contained 0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.2. Samples were mixed (4 : 1) with a denaturing buffer containing 0.05 M sodium phosphate (pH 7.2)/5% SDS/50% glycerol (v/v)/2% bromphenol blue/250 mM dithiothreitol and heated at 100°C for 5 min. Electrophoresis was carried out at 3 mA/gel until the sample entered the gel and then at 6 mA/gel for 6.5 h at 23°C. After electrophoresis, gels were stained for protein with Coomassie brillant blue [11]. Standards used to calibrate the gels were; ovalbumin, bovine serum albumin, bovine liver catalase, bovine α-chymotrypsinogen, carbonic anhydrase and rabbit muscle phosphorylase *a*.

The molecular weight of the oligomer was determined by molecular-sieve chromatography and by density gradient centrifugation. The chromatographic procedure employed a Sepharose 6-B column with a bed vol. of 310 ml (2.5 × 75 cm). The column was standardized against bovine liver catalase, bovine thyroglobulin, horse spleen apoferritin, ovalbumin,

bovine α-chymotrypsinogen and bovine hemoglobin. The density gradient procedure employed a 5–20% linear sucrose gradient prepared in 20 mM succinate buffer (pH 5.5)/0.02% azide. Standards used to define the gradient were porcine kidney D-amino acid oxidase, rabbit muscle lactic dehydrogenase and bovine catalase. Samples were centrifuged in a Beckman SW-41 rotor for 39 h at approx. 39 000 rev./min.

Isoelectric focusing gels

These tube gels were prepared for a broad range pH study (Biolyte pH 3–10) and for a narrow range pH study (Biolyte pH 5–7). Broad range gels contained 5% acrylamide (w/v), 0.13% bisacrylamide (w/v), 4% glycerol (v/v), 4% Biolyte Broad Range pH 3–10 (v/v), 0.15% Temed (v/v) and 0.15% ammonium sulfate (w/v). Narrow range gels were made in the same way except that the pH gradient contained both Biolyte pH 3–10 (1.7%, v/v) and Biolyte pH 5–7 (2.6%, v/v). Samples of enzyme greater than 0.01 units were diluted 1 : 1 with glycerol to a volume no greater than 25 μl. The anode chamber was filled with 0.06 N H_2SO_4 and the cathode chamber with degassed 0.04 N NaOH. The lower chamber was stirred to prevent accumulation of gas bubbles at the tube ends. The electrophoresis was performed at 4°C with a maximum current/gel of 2 mA. After the current has dropped to zero, the gels were electrophoresed at 280 V for 20 h.

The substrate 4-methylumbelliferyl-α-L-fucoside was used as a fluorescent probe for enzyme. Gels were removed from their tubes, rinsed in 0.1 M phosphate buffer (pH 5.5) and placed in a 10-cm gel boat for scanning with a Gilford fluorescent attachment (Model 2515) for the Gilford 240 spectrophotometer. The gels were scanned with phosphate buffer (blank) and with a 4 mM solution of the substrate layered on top of the gel. An intensifying solution of 250 mM glycine (pH 10.4) could be added to stop the reaction and enhance the fluorescence. Enzyme bands could also be seen in the dark with the aid of a hand-held ultraviolet light. The pH gradient of a gel was determined by slicing it into sections after electrophoresis, eluting each section overnight with 1 ml water and measuring the pH of the resulting solutions.

Non-denaturing gels

Acrylamide gels (5%) were prepared using a continuous 0.1 M phosphate buffer (pH 7.9). Samples were mixed 1 : 1 with glycerol and 0.1% bromphenol blue. The samples were electrophoresed at 2 mA/gel for 2 h and then at 4 mA/gel for 12 h at 4°C. Enzyme activity was determined as with isoelectric focusing.

pH optimum determination

The pH optimum of porcine thyroid α-L-fucosidase was determined through the use of a citrate phosphate buffer described by Sober [12] over the pH range from 2 to 9.3. A constant ionic strength was maintained over the pH range.

Active group modification

In carbodiimide studies approx. 0.001 units of enzyme in 0.5 M phosphate buffer at pH 5.5 were incubated with 22 mM CMC and 3 μl bovine serum albumin (1 mg/ml) in the presence and absence of 2.2 M taurine. Aliquots were removed at 0. 15, 30 and 45 min intervals for assay. In studies where substrate protection was examined a lower concentration of CMC was used (2.2 mM) to prevent complete inhibition of the enzyme.

In sulfhydryl reagent studies approx. 0.0005-unit amounts of enzyme were used in 0.5 M phosphate buffer at pH 5.5. With *p*-mercuribenzoate, the effect of the mercurial was examined over a range of 2.5 to 25 nmol for 10 min at 37°C. To determine whether activity could be regenerated by a reducing agent, the inactivated enzyme was incubated with 1 mM dithiothreitol for 20 min at 37°C, and then assayed for fucosidase activity. Protection studies were also conducted with a 0.52 mM concentration of *p*-nitrophenyl glycoside substrate incubated in the presence and absence of *p*-mercuribenzoate. In studies with *N*-ethylmaleimide, 0.65 μM concentrations of the sulfhydryl reagent were incubated with the enzyme for 30 min at 37°C and the enzyme activity then determined. In studies with iodoacetate, 10–600 μM concentrations of that reagent were incubated with enzyme for 30 min at 37°C. Subsequently, residual fucosidase activity was determined.

Thyroglobulin modification studies

Thyroglobulin was subjected to Pronase treatment as described by Spiro [13] and Fukuda and Egami

[14]. Additional samples of the thyroglobulin (5.13 nmol in 700 μl) were treated with 0.2 units neuraminidase for 24 h at pH 5.0 and 37°C. Treated samples were subsequently used as substrates for thyroid α-L-fucosidase.

Enzyme purification procedures

Several separate isolations of α-L-fucosidase were accomplished using the following typical procedure. All operations were conducted at 4°C.

100 g of freshly excised and cleaned porcine thyroid glands (obtained through the courtesy of Ohio Packing Co., Columbus, OH) were minced and then homogenized in an Omni-Mixer with 100 ml 20 mM Mops buffer (pH 7.0) at 4°C. The homogenate was centrifuged in a refrigerated preparative centrifuge at 18 000 × *g*. The supernatant solution was filtered through glass wool to remove lipid materials, diluted with an equal volume of 20 mM Mops buffer and placed on a DEAE-cellulose column previously equilibrated with buffer. The characteristics of the column and the elution regimen are reported in the legend to Fig. 1. Active fractions removed from the column were dialyzed overnight against 4 l 10 mM phosphate buffer (pH 5.5).

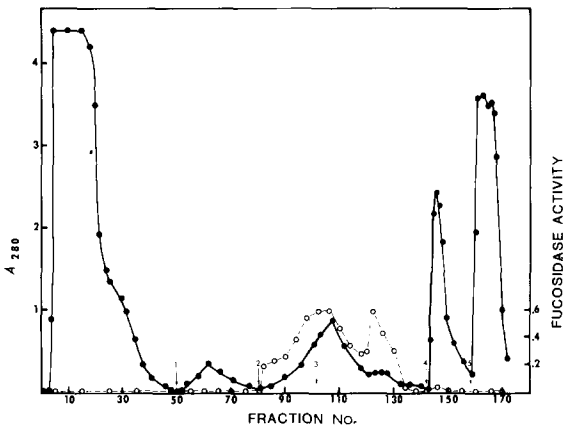

Fig. 1. Protein and enzyme patterns from the first DEAE-cellulose column step. The numbered arrows indicate the initiation of the various elution solutions starting with the 750 ml phosphate buffer wash at narrow No. 1 followed sequentially by a 700 ml linear gradient of 10 mM phosphate buffer (pH 5.8)/0.02% azide/70 mM KCl, a 900 ml wash of 70 mM KCl, a 700 ml wash of 150 mM KCl and a final 350 ml wash with 1 M KCl. 25-ml fractions were collected from the 4.5 × 10 cm column. A_{280} (•———•); fucosidase activity (o – – – o).

One-half of the dialyzed enzyme preparation was passed into an agarose-ε-amino-*N*-caproyl-β-L-fucosamine column previously equilibrated with 10 mM phosphate buffer (pH 5.5). Details of elution procedure are given in Fig. 2. Eluted fractions containing fucosidase activity were pooled and concentrated to a 5 ml vol. in an Amicon concentrator. A precipitate which formed at this point was removed through centrifugation and the remaining solution concentrated to 2 ml.

One-quarter of the affinity concentrate was passed through the P-300 column at time. The aliquot was added to the 55-ml bed vol. column (0.8 × 75 cm) which had been previously equilibrated with 5 mM phosphate buffer (pH 5.8)/0.02% azide. 12-drop fractions (approx. 0.75 ml) were collected as a 40 ml vol. of 5 mM phosphate buffer was added to the column to elute the enzyme.

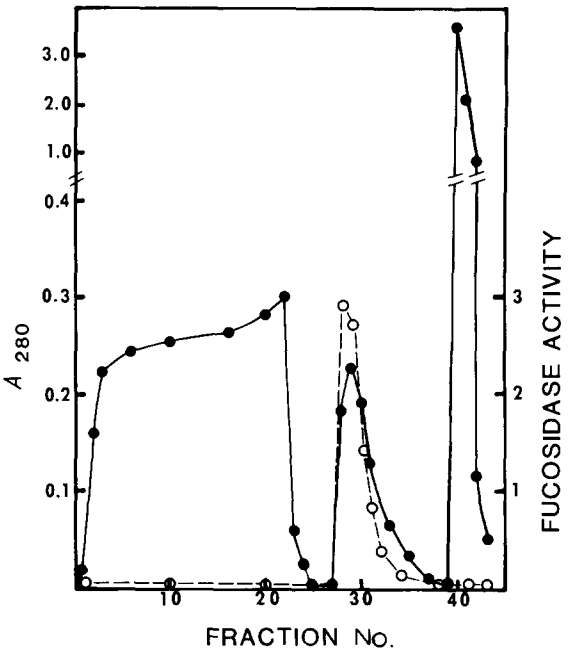

Fig. 2. Protein and enzyme elution patterns from the affinity column step. Column dimensions were 1.6 × 6 cm. After addition of protein the column was washed with 10 mM phosphate buffer (pH 5.5) until the absorbance at 280 nm was less than 0.05 *A* units. Active enzyme was then eluted with 130 ml of 1 mM L-fucose in 20 mM succinate buffer (pH 5.5)/0.02% azide. A final wash with 1 M KCl elutes protein but no fucosidase activity. A_{280} (•———•); fucosidase activity (o – – – o).

The P-300 fractions were pooled and passed into a 1.5 ml bed vol. column (0.8 × 2 cm) of DEAE which had been previously equilibrated with 5 mM phosphate buffer (pH 5.8). 2-ml fractions were collected as the column was eluted successively with 20 ml 5 mM phosphate buffer (pH 5.8), with 20 ml 10 mM phosphate buffer, with 50 ml 20 mM phosphate buffer and with 25 ml 30 mM phosphate buffer. The enzyme activity eluted with the 20 mM phosphate wash. The enzyme fractions were pooled and concentrated to a 1 ml vol. in an Amicon concentrator. The enzyme at this highest stage of purity was quite stable and could be stored at 4°C in 20 mM phosphate buffer with azide for 75 days with only a 15% loss of activity. At −20°C in the same buffer with 40% of added glycerol the enzyme exhibited no loss of activity after 75 days.

Results

Table I presents the purification procedure and its yield for porcine thyroid α-L-fucosidase isolated by the procedures described in Materials and Methods. The overall purification of the enzyme is 10 800-fold.

Samples of α-L-fucosidase derived from each step in the purification process were electrophoresed in acrylamide gels after treatment with SDS. Upon staining with Coomassie brillant blue and destaining, the gels were scanned spectrophotometrically. The process ultimately results in isolation of a single SDS subunit after the second DEAE-cellulose column step.

An important criterion for purity is the specificity of the enzyme preparation relative to its original specificity in the crude extract. The crude extract showed appreciable enzymatic activity toward the nitrophenyl glycosides of α-D-mannose (100%) β-*N*-acetyl-D-galactosamine (100%), α-D-fucose (70%), β-D-glucose (67%), β-*N*-acetyl-D-glucosamine (55%), α-D-galactose (50%), β-D-glucose (37%), β-L-fucose (20%) and α-L-arabinose (13%) as compared to the α-L-fucose glycoside as control (100%). The specificity of the second DEAE-cellulose eluant was narrowed to *p*-nitrophenyl-α-L-fucoside as a substrate with only very slight activities observed with β-*N*-acetyl-D-galactosamine (1.3%) and β-*N*-acetyl-D-glucosamine (2.5%) derivatives.

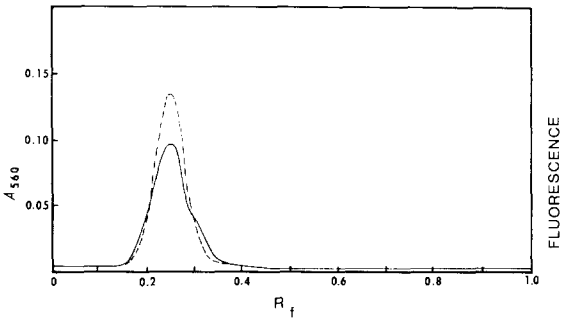

Fig. 3. Optical scans of non-denaturing electrophoresis gel of the final DEAE-cellulose enzyme preparation detecting protein, stained with Coomassie brillant blue (*A*), and α-L-fucosidase activity (fluorescence). A_{560} (———); fluorescence (- - - - - -).

Non-denaturing acrylamide gels were used to assess the purity of the fucosidase preparation obtained from the final DEAE-cellulose column. Fig. 3 contains the optical scans of one of those gels examined for enzymatic activity, as determined by the fluorescence procedure described in Materials and Methods, and for protein concentration utilizing Coomassie brillant blue stain. The two curves appear nearly concentric with a slight shoulder on the protein peak

TABLE I

PURIFICATION TABLE FOR PORCINE THYROID FUCOSIDASE

Purification step	Volume (ml)	Total protein (mg)	Overall yield (%)	Specific activity (units/mg)	Total activity	*N*-Fold purification
10 800 × *g* supernatant	260	43 000	100	0.00072	30	1
DEAE-cellulose (I)	1 000	165	47	0.085	14	120
Fucose affinity column	275	60	44	0.220	13	300
P-300	34	2.7	29	3.2	8.7	4 500
DEAE-cellulose (II)	128	0.4	10	7.8	3.0	10 800

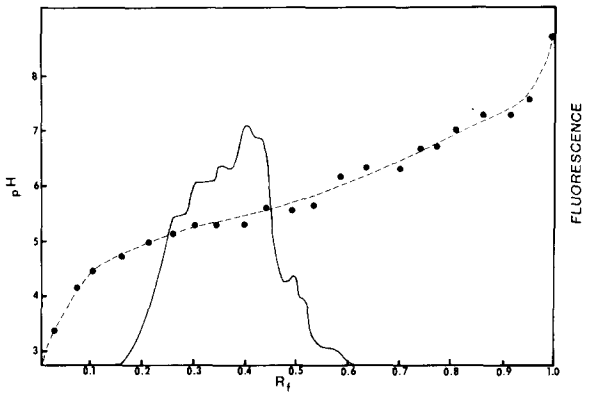

Fig. 4. Fluorescence scan of a gel in which α-L-fucosidase, from the final DEAE-cellulose enzyme preparation, has been subjected to isoelectric focusing and a fluorogenic substrate to demonstrate the microheterogeneity of the enzyme preparation. The broken curve represents the pH gradient for the system.

which suggests a degree of heterogeneity. Since microheterogeneity is commonly observed with glycoprotein enzymes this phenomenon was further examined by the use of isoelectric focusing gels which should accentuate charge differences. Fig. 4 shows a fluorescence scan of isoelectric focusing gels of the highest purity protein after exposure to the substrate 4-methylumbelliferyl-α-L-fucoside as described in Materials and Methods. Fig. 5 is a graphic representation of the gels as seen with a hand-held ultraviolet light. Considerable heterogeneity is apparent with 10 distinct fluorescent bands of differing intensity (Fig. 5A). Since microheterogeneity with glycoproteins is often the result of a variable content of sialic acid residues the enzyme preparation was treated with a neuraminidase preparation from *C. perfringens* and simultaneously electrophoresed. Fig. 5B contains the results of that study. It is apparent that four of the original 10 bands have disappeared with the generation of a single new band of α-L-fucosidase activity. Apparently some of the microheterogeneity is the result of variable numbers of sialic acid residues.

Molecular weight determinations

A standard molecular weight curve was developed for the SDS electrophoresis gels as described. Using this curve and an average R_F value of 0.44 for the SDS subunit of thyroid α-L-fucosidase, a monomer

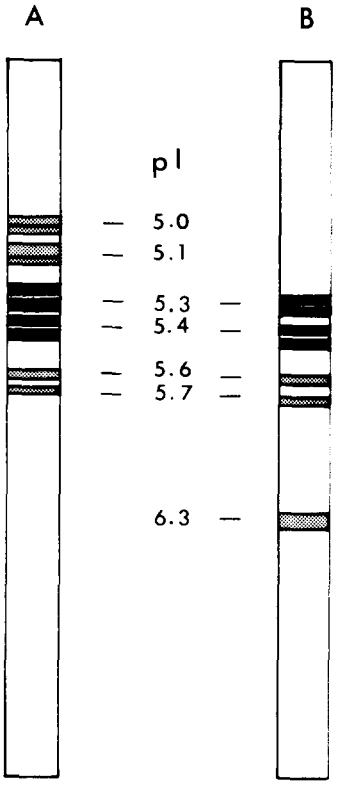

Fig. 5. A graphic presentation of isoelectric focusing gels of α-L-fucosidase exposed to a fluorogenic substrate and an ultraviolet light. Gel B contains the same enzyme preparation (DEAE-cellulose 2) as a gel A but has been treated with neuraminidase prior to electrophoresis. Where two enzyme bands are almost overlapping an average p*I* is calculated for the pair. Major bands are represented as black while minor bands are stippled.

TABLE II

FUCOSIDASE MOLECULAR WEIGHT DETERMINATIONS

Technique	Molecular weight	Number of trials
SDS	55 000 ± 2 000 220 000 ± 8 000 (calculated tetramer)	6
Sepharose 6-B	255 000 ± 5 000	2
Sucrose density gradients	192 000 ± 3 000	2

molecular weight of 55 000 was obtained (Table II). In addition to a subunit value an oligomer molecular weight determination was obtained through gel filtration on a Sepharose 6-B column and through sucrose density gradient experiments. A significant difference in the oligomer molecular weight value is obtained by these different methods (Table II).

pH optimum

A pH optimum curve was established by the procedure described in Materials and Methods. This curve contains a single optimum peak with a narrow plateau at pH 5.1. Considerable enzyme activity remains, however, (>50%) at pH values from 4 to 6.5.

Substrate specificity

Thyroid α-L-fucosidase is highly specific for the carbohydrate moiety and for the nature of the bond to the aglycone. It is, however, much less specific with respect to the aglycone itself since it will accept, as substrates, both the *p*-nitrophenyl- and the 4-methylumbelliferyl-α-L-fucoside. It does not, of course encounter those substrates in the cellular milieu. Rather, it hydrolyses fucoside residues attached to other saccharide units. In order to partially assess the specificity of the fucosidase toward its more 'normal' substrates we examined its effects on oligosaccharides available to us. These included a trisaccharide from porcine submaxillary mucin in a ^{3}H-labeled and unlabeled form (Fucα1 → 2 Galβ1 → 3 Gal-OLNAc), a lacto-*N*-fucopentaose I (Fucα1 → 2 Galβ1 → 3 GlcNAcβ1 → 3 Galβ1 → 4 Glc) a lacto-*N*-pentaose II (Galβ1 → 3(Fucα1 → 2) GlcNAcβ1 → 3 Galβ1 → 4 Glc), and porcine thyroid thyroglobulin. Since supplies of these potential substrates were only available in small quantities through the generosity of other investigators, saturating kinetics could not be employed. However, qualitative assessments of their efficacy as substrates could be derived. Table III contains data obtained from hydrolysis studies with the three oligosaccharides. The mucin trisaccharide appears to be the most effective of the three substrates with lacto-*N*-fucopentaose II significantly more effective than the corresponding lacto-*N*-fucopentaose I. All substrates were, however, attacked by the thyroid fucosidase to some degree.

The action of the enzyme of porcine thyroglobin is also shown in the data of Table III. The fucosidase appears virtually without hydrolytic effect on the untreated glycorprotein. Prior treatment with neuraminidase makes the resulting degraded thyroglobulin more susceptible to α-L-fucosidase. It should be noted, however, that the neuraminidase blank values are considerably higher with respect to free L-fucose than the original thyroglobulin sample. Consequently it might be suspected that the neuraminidase is removing more than sialic acid residues from the substrate. Thus more extensive proteolytic or glycolytic reduction of the substrate might be occurring. Pronase treatment of thyroglobulin releases very little

TABLE III

THE ACTION OF THYROID FUCOSIDASE ON 'NATURAL' SUBSTRATES

Substrate	Prior treatment	Enzymatic hydrolysis (%)	Blank-free L-fucose (%)	Net-hydrolysis of L-fucose residues (%)
Submaxillary mucin trisaccharide	None	82.3 ± 9.7 (3) [a]	0.6 ± 0.72 (3)	81.7
Lacto-*N*-fucopentaose I	None	19.5 ± 3.5 (2)	10.7 ± 1.8 (2)	8.8
Lacto-*N*-fucopentaose II	None	38.0 (1) [b]	1.3 (1)	36.7
Thyroglobulin	None	4.0 ± 0.4 (4)	1.9 ± 0.5 (2)	2.1
Thyroglobulin	Neuraminidase	21.7 ± 0.8 (4)	10.0 ± 1.0 (4)	11.7
Thyroglobulin	Pronase	40.0 ± 5.0 (4)	2.1 ± 0.5 (4)	37.9

Thyroglobulin samples were incubated with 0.03 units fucosidase at 37°C for 24 h all other substrates were incubated with 0.5 units fucosidase. A thyroglobulin molecular weight of 670 000 was assumed for these studies [27].

[a] Standard deviation and number of assays.

[b] Limited supplies of substrate restricted numbers of assays.

additional L-fucose suggesting only proteolytic reduction of the substrate. This Pronase-treated thyroglobulin is, however, much more susceptible to α-L-fucosidase attack.

Inhibitory monosaccharides

A number of monosaccharides posses structural similarities to α-L-fucose and thus they, and free L-fucose itself, might prove to be effective inhibitors of thyroid α-L-fucosidase. The free sugars L-fucose, L-galactose, D-arabinose and L-rhamnose were all examined in this regard and shown to inhibit thyroid α-L-fucosidase to varying degrees with K_i values of 0.13, 1.7, 50 and 140 mM, respectively, as compared to a K_m values of 0.3 mM for the substrate *p*-nitrophenyl-α-L-fucoside. Clearly, free L-fucose functions most effectively as an α-L-fucosidase inhibitor. The character of the inhibition of these enzymes was shown to be of the competitive type in all cases by the use of Lineweaver-Burk plots.

Enzyme inactivation-group specific reagents

Several compounds were used to investigate the enzyme's susceptibility toward sulfhydryl reagents. Treatment of thyroid α-L-fucosidase with *N*-ethylmaleimide and iodoacetate was without effect at concentrations of reagent between 50 nM to 60 μM for the former and 10–600 μM for the latter. The enzyme was, however, completely inactivated by *p*-mercuribenzoate at a 250 nM concentration. 50% inactivation was achieved at a 25 nM concentration of the mercurial. Incubation of the enzyme with *p*-mercuribenzoate and *p*-nitrophenyl-α-L-fucoside did not protect against the inhibitor and subsequent incubation with 1 mM dithiothreitol did not regenerate a significant amount of enzyme activity.

Detection of essential carboxyl groups was achieved by the use of a water-soluble carbodiimide, 1 cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate. This reagent, at a 22 mM concentration, causes a complete inactivation of the enzyme (Fig. 6A). Inactivation remains the same whether or not taurine is added as a nucleophile to displace the carbodiimide group and form a stable bond with the carboxyl group [15]. The effect of adding the substrate *p*-nitrophenyl-α-L-fucoside prior to treatment with CMC is shown in Fig. 6B. The substrate appears to protect the enzyme so that only

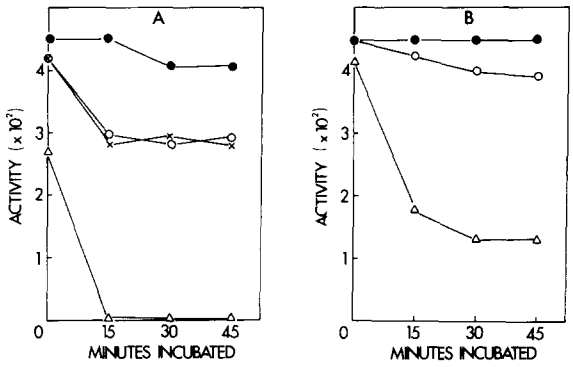

Fig. 6. Influence of a free carboxyl group modifying reagent on the activity of α-L-fucosidase. Graph A demonstrates the inhibitory action of a water soluble carbodiimide on the enzyme. ●———●, 0.0 mM CMC; ○———○, 2.2 mM CMC; ×———×, 2.2 mM CMC + 2.2 mM taurine and △———△, 2.2 mM CMC. Graph B demonstrates that the substrate *p*-nitrophenyl-α-L-fucoside protects the enzyme against carbodiimide action, whereas the β-D-analogue is without protective effects. In graph B the closed circles represent enzyme assayed after preincubation in the absence of CMC for various time intervals. The open circles represent enzyme assayed after preincubation with 2.2 mM CMC and 0.625 mM *p*-nitrophenyl-α-L-fucoside. The triangles represent enzyme assayed after preincubation with 2.2 mM CMC and 0.625 mM *p*-nitrophenyl-β-D-fucoside.

1–2% of the activity is lost. A possible explanation of these data is that the substrate may not be protecting against CMC by occupying the active site but rather interacting with CMC directly and inactivating it. In order to examine this possibility protection was attempted with *p*-nitrophenyl-β-D-fucoside, an ineffective substrate for the enzyme (since it shows little affinity for the active site). Fig. 6B shows that this substrate is completely ineffective in protecting the enzyme from CMC.

In addition to reacting with carboxyl groups, compounds such as CMC are known to interact with tyrosine residues [16]. These substituted tyrosine residues are susceptible to hydrolysis by hydroxylamine to restore the tyrosine residue and hence the activity of the enzyme. No reversal of the inactivation of the enzyme was however observed in the presence of hydroxylamine.

Discussion

α-L-Fucosidases have been isolated from several tissues by one or more passages through a fucosyl-

affinity column [6,17–20]. Porcine thyroid tissue proved refractory to that approach and required several sizing and charge separation procedures to ultimately purify the enzyme. It is possible that the glycoprotein thyroglobulin, which represents 80% of the protein of thyroid tissue [21], may interact with affinity groups rendering them far less effective.

The only porcine α-L-fucosidase reported to date has been that from kidney tissue. Wiederschain and Rosenfeld [22] in a preliminary communication, reported that two separate α-L-fucosidase activities could be detected when pig kidney extracts were eluted from a Sephadex G-200 column. They reported one of the activities to be approx. one-half the molecular weight of the other but no molecular weight values were specified. Although some difference in specificity toward substrates exist it is possible that the two forms indentified may simply represent molecular aggregates as reported for other α-L-fucosidases [7,19,20]. Too little information is available for the pig kidney enzyme to permit a correlation with the thyroid enzyme. Our isolation procedures did not detect two species differing in molecular weight by a factor of two but isoelectric focusing did reveal considerable microheterogeneity in the final product. Four of the ten enzyme bands detected by this device appeared to be heterogeneous with respect to sialic acid residues. The six remaining bands, including the four major bands may be without sialic acid residues or, alternatively, may have them hidden from neuraminidase action. Other explanations of microheterogeneity may be related to variable amidation of free carboxyl groups or limited proteolysis during enzyme isolation. Although different with respect to the absolute specifics of number of major and minor bands and isoelectric points, the pattern of isoelectric focusing in human liver [17,23] is similar to that of thyroid tissue. In both cases, minor, more acidic bands are neuraminidase sensitive, whereas the major bands are not. This is in sharp contrast to human sera [18] where the major bands are neuraminidase sensitive.

The molecular weight values obtained for thyroidal α-L-fucosidase vary extensively as a function of the technique used to measure them. Glycoprotein subunit molecular weight values obtained by electrophoresis in SDS-polyacrylamide gels are generally greater than their true value since the glycone portion of the molecule does not bind SDS and does not migrate as rapidly as it would if the structure were all protein [24]. Thus, the 55 000-dalton value for the subunit is probably significantly elevated. Since the molecular-sieve method is also prone to error due to interaction between the glycoprotein and the polysaccharide sieve we tentatively support 190 000 daltons, the sucrose gradient value, as the most reasonable estimate of the molecular weight. If the oligomer is tetrameric, this would provide a subunit molecular weight of 47 500. These values are not disparately different from those reported for other α-L-fucosidases [6,17,18,20].

Active site studies with sulfhydryl reagents showed little inactivation of the enzyme with *N*-ethylmaleimide and iodoacetate but extensive inhibition with *p*-mercuribenzoate which was not protected by substrate. These data may indicate that sulfhydryl groups are important in maintaining the confirmation of the protein but are not present at the active site. Carbodiimide studies suggest a carboxyl group(s) as present and important at the enzyme active site. Its detection is consistent with the pH optimum of the enzyme and the fact that carboxyl groups are found at the active site of other glycosidases. Lysozyme is the classical example of such an enzyme and presumably the carboxyl group(s) of α-L-fucosidase may play a similar mechanistic role to that proposed for lysozyme [25].

Specificity studies with thyroidal fucosidase suggest that the enzyme, as with other fucosidases, is highly specific for the L-sugar and that the bond must be of the α configuration. The nature of the other group attached to that bond is far less absolute since the enzyme accepts both *p*-nitrophenyl- and 4-methylumbelliferyl groups readily in spite of the considerable differences in their structure. Specificity studies with oligosaccharide are too fragmentary to provide a complete picture of which sugars are important in their attachment to L-fucose. The difference in susceptibility to attack of lacto-*N*-fucopentaose I as compared to lacto-*N*-fucopentaose II suggests that the point of attachment of the L-fucose residue is of some significance. This may be due to the configuration of the non L-fucose moiety as suggested by Wiederschain and Rosenfeld for the liver enzyme [22]. Alternatively, the enzyme might possess an L-fucose recognizing site and a hydrophobic site for

the molecule to which L-fucose is attached. *N*-Acetylglucosamine or similar residues directly attached to L-fucose may serve effectively as the less polar moiety needed in that interaction. Such a hydrophobic site has been proposed for human α-L-fucosidase [26].

The data obtained in studies with thyroglobulin as a substrate point to the role of thyroidal α-L-fucosidase as a salvage enzyme for recovery of L-fucose from the molecule. In that salvage process the enzyme does not attack the intact thyroglobulin directly. Considerable degradation of the glycoprotein must occur with other enzymes, including proteolytic removal of the majority of the aglycone protein, before the oligosaccharide is suitable as a substrate.

Acknowledgement

The authors wish to acknowledge financial support for this work through a grant from the National Science Foundation (PCM 7911032).

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