Purification and Properties of an α-L-Fucosidase from Rat Epididymis

ROBERT B. CARLSEN and JOHN G. PIERCE
From the Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, California 90024
(Received for publication, June 24, 1971)

SUMMARY

An α-L-fucosidase has been purified from rat epididymis by fractionation of extracts with ammonium sulfate and subsequent chromatography on ion exchange celluloses. It is a glycoprotein with a molecular weight of 210,000 to 220,000 and dissociates in the presence of mercaptoethanol and guanidine hydrochloride or sodium dodecyl sulfate. The dissociated material behaves as two components with molecular weights of approximately 47,000 and 60,000, thus suggesting two pairs of dissimilar subunits. A preliminary amino acid and carbohydrate composition is reported.

Other glycosidases of rat epididymis, α-D-mannosidase, β-D-N-acetylgalcosaminidase, and β-D-galactosidase have been partially purified from the same extract. All were active against a variety of glycoprotein and glycopeptide substrates. The purified fucosidase preparation is stable, is essentially free from other glycosidases with the exception of small amounts of N-acetylglucosaminidase activity, and it catalyzes the hydrolysis of p-nitrophenyl-α-L-fucoside. Studies with glycopeptides show quantitative release of fucose by the purified enzyme from the tryptic glycopeptide of the hormone-specific chain of bovine luteinizing hormone and from a peptide of horse immunoglobulin G.

A general method has been developed for quantitative determination by gas-liquid chromatography of the monosaccharides released by enzymic hydrolysis of glycoproteins and glycopeptides.

The structures of the complex oligosaccharides found in glycoproteins are of considerable interest. Studies of structure have been hindered, however, by the limitations of chemical methods such as partial acid hydrolysis, methylation, and oxidation with periodate. Recently, a number of enzymes which degrade these oligosaccharides have been isolated (1-9). Such enzymes are of considerable interest. Studies of structure have been hindered, however, by the limitations of chemical methods such as partial acid hydrolysis, methylation, and oxidation with periodate. Recently, a number of enzymes which degrade these oligosaccharides have been isolated (1-9). Such enzymes are of considerable interest.

EXPERIMENTAL PROCEDURE

Materials—p-Nitrophenyl-α-L-fucopyranoside, p-nitrophenyl-α-D-mannopyranoside, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucofuranoside, and p-nitrophenyl-β-D-galactopyranoside were obtained from Pierce Chemical Co., Rockford, Ill. Carrier ampholytes were obtained from LKB Instrument Co., Rockville, Md. Whatman CM-2 and DEAE-celluloses were obtained from Reeve Angel Co., Clifton, N.J.; Cellex-D DEAE-cellulose was obtained from Guest, N.J.

1 Fucose refers to L-fucose throughout. Reference to all other sugars is to the β isomer.

2 The abbreviations used are: CM-, carboxymethyl-; SE-, sulfoethyl-; SDS, sodium dodecyl sulfate; LH, luteinizing (interstitial cell-stimulating) hormone; I.H., the hormone-specific chain of LH.
from Bio-Rad Laboratories, Richmond, Ca.; SE-Sephadex and Sephadex gel filtration media were from Pharmacia, Piscataway, N. J.; and Bio-Gel P-10 was from Calbiochem, Los Angeles.

Human, horse, and rabbit immunoglobulins (Fraction II) and bovine serum albumin (Fraction V) were obtained from Pentex, Kankakee, Ill., trypsin (twice crystallized), pepsin (twice crystallized), and carboxypeptidases A and B (disosopropyl fluorophosphate-treated) from Worthington, Freehold, N. J., and amino- and carboxypeptidase M from Henley and Co., New York.

Bovine luteinizing hormone and its β chain (formerly designated CII) were prepared by the procedures of Liao et al. (20). The immunoglobulin glycopeptides were prepared by tryptic hydrolysis of the reduced, S-carboxymethylated proteins. The hydrolysates were fractionated by gel filtration on Sephadex G-50 (fine) in 1 N acetic acid followed by countercurrent distribution of the glycopeptide peaks by the "fundamental" procedure of Craig (21) with 30 transfers with the solvent system of Rawitch et al. (22) and Howard and Pierce (23). Exposure to acid was minimized by immediate neutralization of the tubes upon completion of the distribution. A homologous set of glycopeptides, designated Fe peptides, was obtained from horse, human, and rabbit immunoglobulins. These peptides originate in the carboxyl-terminal segment of the heavy chains and the amino acid sequences of two of them, human and rabbit, have been reported (24, 25). A second type of tryptic glycopeptide was obtained only from the horse material and is designated horse L peptide as it may originate in the light chains. Its composition and size are very similar to those reported by Melchers (26) for a light chain glycopeptide from a mouse myeloma protein. The LH-β glycopeptides were prepared either by hydrolysis of LH-β with pepsin and subsequent hydrolysis with amino- and carboxypeptidase M and carboxypeptidases A and B or by hydrolysis with trypsin. The peptic hydrolysate was fractionated by gel filtration on Sephadex G-50 (fine) in 1% NH₄HCO₃. The tryptic hydrolysate was fractionated on Bio-Gel P-10 (200 to 400 mesh) in 0.5% NH₄HCO₃ followed by countercurrent distribution by the "single withdrawal" procedure of Craig (21) with two tubes and ten transfers with the solvent system previously cited (22, 23).

Enzyme Assays—A suitable aliquot (5 to 25 μl) of the enzyme solution is diluted to 100 μl with the same buffer and incubated at 37° for 10 min before addition of 100 μl of substrate solution, also at 37°. The reaction is allowed to proceed at 37° from 1 to 60 min depending on enzyme concentration and then stopped by addition of 2.0 ml of 0.1 M sodium glycinate, pH 10.6. The substrate solutions are as follows: for α-L-fucosidase, 3 mM p-nitrophosphoryl-α-L-fucopyranoside in 0.5 M sodium citrate, pH 6.5; for α-D-mannosidase, p-nitrophosphoryl-α-D-mannopyranoside (saturated solution at 4°) in 0.5 M sodium citrate, pH 5.0; for β-N-acetylglucosaminidase, p-nitrophosphoryl-2-acetamido-2-deoxyβ-D-glucopyranoside (saturated solution at 4°) in 0.5 M sodium acetate, pH 5.0; and for β-D-galactosidase, 10 mM α-nitrophosphoryl-β-D-galactopyranoside in 0.5 M sodium acetate, pH 3.75. All substrate solutions contain 0.02% NaN₃. The released p-nitrophosphoryl compound is determined by measurement of the absorbance at 410 nm; where α-nitrophosphoryl is released, absorbance is measured at 425 nm. One unit of enzyme activity is defined as that amount which will hydrolyze 1 μmol of substrate per min. Protein was determined by the method of Lowry et al. (27) with bovine serum albumin as standard.

Determination of Glycosidase Stabilities—The stabilities of the four enzymes with respect to pH were determined at 37 and 70°. The enzyme fractions used are described below and are α-fucosidase, Fraction DEAE-III; α-mannosidase, 50 to 60% ammonium sulfate fraction; β-D-acetylgalactosaminidase, Fraction DEAE-I; and β-D-galactosidase, Fraction CM-I.

Measurements of pH stability were carried out by diluting 5-μl aliquots of each enzyme solution to 100 μl with buffer to give the proper pH value for incubation; 0.10 M sodium citrate, 0.02% in NaNO₃, was used between pH 2 and 7 and 0.05 M Tris-HCl between pH 7 and 10. The pH values of the incubation mixtures, including the standard substrate solutions, were measured and corrections of the activity made based on the pH optimum curve for each enzyme (see Fig. 4). For the study at 37°, the diluted enzyme was placed in a water bath at 37° for 2 hours before assay. For the study at 70°, the enzyme was incubated at 70° for 15 min and then transferred to a water bath at 37° 10 min before assay.

Stability of the enzymes under conditions which obtain during a glycoprotein or glycopeptide hydrolysis was also determined, except that the enzymes were 10 to 20 times more dilute than during an actual hydrolysis. The diluted enzymes were incubated at 37° for periods of 0 to 200 hours prior to assay; the latter time is the maximum used in hydrolysis of glycopeptides. Dilutions were as follows. The α-fucosidase was diluted to 0.4 unit per ml (0.03 mg of protein) in 0.1 M sodium citrate, pH 6.0; the α-mannosidase was diluted to 0.9 unit per ml (0.75 mg of protein) in 0.1 M sodium citrate, pH 6.0; the β-D-acetylgalactosaminidase was diluted to 1 unit per ml (0.25 mg of protein) in 0.1 M sodium acetate, pH 5.5; and the β-D-galactosidase was diluted to 0.8 unit per ml (1.5 mg of protein) in 0.1 M sodium acetate, pH 4.7. All buffers contained 0.02% NaN₃. Stability of the glycosidase activities in the 35 to 50% ammonium sulfate fraction, which contains all of the enzymes, was also determined.

Determination of pH Optima—Enzyme (35 to 50% ammonium sulfate fraction) and substrate solutions were made up in 0.05 M ammonium acetate, pH 6.2, and the pH values of the substrate solutions adjusted with HCl or NaOH so as to give the appropriate assay pH values when mixed with an equal volume of enzyme solution. Both enzyme and substrate solutions were initially incubated at 37° for 10 min and then 0.1-ml volumes of each were mixed. Assays were for 2 min.

Extraction and Concentration of Enzymes—Crude extracts of the rat epididymal glycosidases were prepared by modifications of the procedure of Conchie and Hay (11). Epididymides were obtained from adult rats (Sprague-Dawley), 10 to 15 weeks old, the adhering fat removed, and the tissue stored in 30% glycerol at 20° until needed. The tissue can be stored in this manner for at least 2 years without significant losses in the yields of enzyme. From 15 to 25 g of epididymal head sections, wet weight, (Sections 1 to 6, Reference 28) were chopped in cold 0.1 M sodium acetate-0.1 M sodium chloride, pH 6.0 (10 ml per g of tissue, wet weight) for 75 s in a Waring Blender, alternating low (8,000 rpm) and high (15,000 rpm) speeds every 15 s, low speed first. The material was then homogenized in an all glass homogenizer, heated to 37°, and held at that temperature for 1 hour before releasing the enzymes, which are lysozymes (10). The pH was then immediately adjusted to 5.25 with glacial acetic acid and the mixtures heated to 60° for 10 min before being cooled to room temperature in an ice bath. The mixture was then centrifuged at 80 °C for 30 min at 35,000 × g, the precipitate discarded, and the pH
of the supernatant adjusted to 6.3 with 15 × NH₄OH. Solid ammonium sulfate was added to the supernatant to 35% of saturation. The solution was allowed to stand at 4°C for 3 hours, then centrifuged at 0°C for 30 min at 29,000 × g. The precipitate was discarded and the supernatant brought to 50% of ammonium sulfate saturation by addition of solid ammonium sulfate and allowed to stand at 4°C for 20 hours. It was then centrifuged at 0°C for 30 min at 29,000 × g. The supernatant was brought to 60% of ammonium sulfate saturation by addition of solid ammonium sulfate and allowed to stand at 4°C for 20 hours. It was then centrifuged at 0°C for 30 min at 29,000 × g. The precipitate was dissolved in 2.0 ml of 0.1 M sodium citrate, pH 6.0, 0.02% in NaNO₃ to yield the 35 to 50% ammonium sulfate fraction. The supernatant was brought to 50% of ammonium sulfate saturation with solid ammonium sulfate, and the resulting suspension centrifuged at 0°C for 30 min at 29,000 × g after standing at 4°C for 24 hours. The precipitate was dissolved in 0.1 M sodium citrate, pH 6.0, 0.02% in NaNO₃. For recovery of material after chromatography on DEAE-cellulose, pooled fractions were adjusted to pH 6 with glacial acetic acid, brought to 95% saturation with solid ammonium sulfate, and the resulting suspension centrifuged at 0°C for 30 min at 33,000 × g after standing at 4°C for 24 hours. The fucosidase was again dissolved, for storage, in the citrate buffer, pH 6.0; the other fractions were dissolved in 0.1 M sodium acetate, pH 5.5.

**Molecular Weight Determinations on Purified α-fucosidase**

The molecular weight was determined by high speed sedimentation equilibrium in a three-channel interference cell in the Spinco model E ultracentrifuge, equipped with interference optics, by the meniscus depletion method of Yphantis (29). Half and whole fringes were read. Protein concentrations were from 0.36 to 0.80 mg per ml and all determinations were made at 20°C. A value of 0.728 ml per g, based on the composition, was calculated for the partial specific volume. The native material was run in 0.1 M sodium citrate, pH 6.0, 0.02% in NaNO₃ at a rotor speed of 12,690 rpm. To determine the subunit molecular weight the sample was run in 0.05 M sodium barbital-0.65 M NaCl, pH 7.2, 6 M guanidine-HCl, and 0.5% in β-mercaptoethanol. The initial rotor speed was 36,000 rpm, slowed to 30,000 rpm after 22 hours.

**Electrophoresis**—Electrophoresis of the native hormone was carried out at pH 9.5 in Tris-phosphate buffer with a glycine-running buffer as described by Liao et al. (20). For the 5.6% acrylamide gel, pH 6.6, the concentrations of all reagents except the ammonium persulfate were reduced appropriately. The molarity of the Tris buffer was 0.095. Enzyme activity was located after electrophoresis at pH 6.6 by cutting the gel into segments; each segment was macerated and allowed to stand 100 µl of citrate buffer, pH 6.6, 0.1 M, for 15 min at 37°C. Substrate was added (100 µl of 3 max p-nitrophenyl fucoside) and the solution again allowed to stand for 15 min. Full color was then developd by addition of alkali.

The enzyme was also subjected to electrophoresis on 5% polyacrylamide gels in the presence of 0.1% SDS according to the method of Weber and Osborn (30). Electrophoresis was at 6 mA per tube (27 volts) for 74 hours at room temperature. Standards run concurrently for molecular weight determination were peptisin, ovalbumin, bovine serum albumin, glutamic dehydrogenase, Escherichia coli β-galactosidase, and thyroglobulin. Two determinations were made with the α-fucosidase; one with 20 µg of material and the other with 30 µg.

**Hydrolysis of Glycoprotein and Glycopeptide Oligosaccharides with Rat Epididymal Glycosidases**—Hydrolases for 72 hours were carried out with a crude enzyme mixture (35 to 50% ammonium sulfate fraction) and the time course of hydrolysis was determined with the purified α-fucosidase (Fraction DEAE-III). From 25 to 75 nmoles of protein or peptide sample were used per single hydrolysis or per 100-µl aliquot for time study hydrolyses.

For 72-hour hydrolyses with the crude enzyme, the total amounts of each enzyme per hydrolysis were α-fucosidase, 1 unit; α-mannosidase, 1.5 units; β-N-acetylgalactosaminidase, 2.0 units; and β-galactosidase, 0.75 unit. These amounts were the sum of three equal additions of enzymes at 0, 24, and 48 hours. For hydrolyses with the purified α-fucosidase sufficient enzyme was added so that 0.5 unit of α-fucosidase was present per 100-µl aliquot. The total amount of enzyme was added at 0 hour.

The substrate was dissolved in a solution containing the internal standard needed in the subsequent sugar determinations by gas-liquid chromatography (20 µg of 2-deoxyglucose per 25 µl in glass-distilled water) so that 20 µg of inositol were present per hydrolysis or per aliquot. The enzyme solution was then added and the mixture diluted, if necessary, to 100 µl per hydrolysis or per aliquot. The enzyme solution was then added and the mixture diluted, if necessary, to 100 µl per hydrolysis or per aliquot. The enzyme solution was then added and the mixture diluted, if necessary, to 100 µl per hydrolysis or per aliquot.

Hydrolases were at 37°C with the enzymes added and dilutions made in 0.1 M sodium citrate, pH 6.0, 0.02% in NaNO₃.

**Isolation and Determination of Monosaccharides Released from Glycopeptides by Glycosidases**—To isolate the released monosaccharides the hydrolysate was dialyzed with water to about 15 ml in an Amicon model 10-PA ultrafiltration cell and ultrafiltered through a PM-10 membrane, 10,000 molecular weight cutoff (Amicon Corp., Lexington, Mass.), to remove most of the protein. The retentate was discarded and the ultrafiltrate concentrated to about 1 ml by rotary evaporation, then passed through three columns, 0.5 ml each, of Dowex 50-X12 (200 to 400 mesh) (H⁺ form), the second 300 mg of Dowex 2-X8 (200 to 400 mesh) (OH⁻ form), and the third a 0.1-ml bed of SE-Sephadex C-25 (H⁺ form). The columns were washed three times with 1.0 ml of water each time. This treatment desalts the mixture and removes any glycopeptide substrate or remaining protein. The eluate was collected and concentrated to about 0.5 ml by rotary evaporation, redissolved in 0.5 ml of water, and passed through about 200 mg of Dowex 50-X12 (200 to 400 mesh) (H⁺ form) to remove the free hexosamines which are later eluted from the resin with 3 ml of 6 M HCl and analyzed on an amino acid analyzer. The neutral
sugars are converted to their alditol acetates and analyzed by gas-liquid chromatography by the method of Kim et al. (31).

RESULTS

Purification of Glycosidases—The procedure results not only in purification of the \( \alpha \)-fucosidase but separates the other activities reasonably well. The results are summarized in Table I. The fucosidase, essentially free of the other glycosidase activities, except for \( N \)-acetylglucosaminidase activity, was found to be in the last fraction eluted from DEAE-cellulose. Based on the activity of the homogenate, a 180-fold purification was achieved (12% yield of fucosidase activity).

Addition of 0.1 M \( \text{NaCl} \) to the extraction buffer greatly improves the yield of \( \alpha \)-mannosidase as reported by Snait and Levvy (12). The yield of \( \alpha \)-mannosidase is also sensitive to the pH of the extraction buffer, the optimum being at about pH 6. The yields of the other enzymes are not appreciably affected by these parameters in the pH range 5 to 6. The step of heating at 60°C precipitates approximately half of the protein present in the 37°C homogenate. Adjustment of the pH to 5.25 prior to heating at 60°C is a compromise which gives the best yields for all four enzymes. It was also found that adjustment of the pH to 6.3 improved the ammonium sulfate fractionation and increased the amount of \( \alpha \)-mannosidase precipitating between 50 and 60% saturation.

In order to separate the \( \alpha \)-fucosidase, \( \beta \)-\( N \)-acetylglucosaminidase, and \( \beta \)-galactosidase, the 35 to 50% ammonium sulfate fraction is chromatographed on CM-cellulose. The results are shown in Fig. 1a. The \( \beta \)-galactosidase was recovered from Fraction CM-I and was contaminated with about 12% \( \alpha \)-fucosidase and small amounts of the other enzymes (based on activity against nitrophenyl glycoside substrates). Fraction CM-II contained the \( \alpha \)-fucosidase and \( \beta \)-\( N \)-acetylglucosaminidase and was rechromatographed on DEAE-cellulose. The \( \alpha \)-mannosidase activity is largely lost during the chromatography on CM-cellulose but that remaining (less than 10%) also emerges in this fraction. The results of the chromatography on DEAE-cellulose are shown in Fig. 1b. It can be seen that, as measured by absorbance at 280 nm, most of the protein emerged with the solvent front (Fraction I) which also contained the \( \beta \)-\( N \)-acetylglucosaminidase contaminated with \( \alpha \)-mannosidase and \( \beta \)-\( N \)-acetylglucosaminidase contaminated with \( \alpha \)-mannosidase and \( \beta \)-galactosidase. The protein peak corresponding to the fucosidase was just detectable by the absorption meter used (Gilson UV-280 IF) but coincided with the enzymic activity.

Inactivation of Contaminating Activities—Each enzyme preparation obtained by chromatography is contaminated with varying amounts of other activities. Therefore, inactivation of the contaminants was attempted. The best treatment found thus far for each enzyme and the results obtained are summarized in Table II. Treatment of the \( \beta \)-galactosidase for 2 hours at 37°C in 0.1 M sodium citrate, pH 3.25, 0.02% in \( \text{NaN}_3 \) (see Fig. 2a) is sufficient to inactivate most of the contaminating activities with 60% recovery of \( \beta \)-galactosidase activity. The \( \beta \)-\( N \)-acetylglucosaminidase is less sensitive to inactivation by cupric acetate than are the other enzymes and treatment with 5 mM cupric acetate in 0.1 M sodium acetate, pH 5.5, 0.02% in \( \text{NaN}_3 \) (2 mM copper concentration) at 25°C overnight inactivates much of the contaminating activity in Fraction DEAE-I with no loss of \( \beta \)-glucosaminidase activity. At pH 6 the \( \alpha \)-fucosidase is more heat stable than are the other enzymes (see Fig. 2b). Heating of this enzyme at 70°C for 15 min in 0.1 M sodium citrate, pH 6.0, 0.02% in \( \text{NaN}_3 \) inactivates much of the contaminating activities remaining in the preparation, particularly \( \beta \)-\( N \)-acetylglucosaminidase, while 90% of the \( \alpha \)-fucosidase activity is recovered. The \( \alpha \)-mannosidase is obtained in the 50 to 60% ammonium sulfate fraction and although contaminating \( \alpha \)-fucosidase was easily inactivated by treatment with 5 mM \( \text{ZnCl}_2 \) in acetate buffer, repeated attempts to inactivate the other activities were unsuccessful. While it would seem feasible to inactivate both \( \beta \)-galactosidase and \( \beta \)-\( N \)-acetylglucosaminidase with good recovery of \( \alpha \)-mannosidase by heating at 70°C in citrate buffer at pH 6.0 or 6.5 (see Fig. 2b), attempts to do so with the undiluted enzyme in both citrate and acetate buffers resulted in almost complete loss of \( \alpha \)-mannosidase activity as well as of the other activities.

Glycosidase Stabilities—The stabilities of the four enzymes with respect to pH were determined at 37 and 70°C on the several fractions and the results are shown in Fig. 2. Enzyme stability was
Fig. 1. \( a \), chromatography of the 35 to 50\% ammonium sulfate fraction (410 mg of protein) on CM-cellulose (Whatman CM-32, microgranular). The column, 2.4 x 28 cm, was run at room temperature at a flow rate of 60 to 70 ml per hour; 10-ml fractions were collected. The column was developed initially with 350 ml of 0.011 M citric acid-NaOH, pH 5.50, 0.010 M NaCl, and then a linear gradient over 1800 ml to 0.25 M NaCl in the same buffer was applied to complete development. Aliquots of 25 \( \mu \)l of the effluent were assayed for \( \alpha \)-fucosidase (---), \( \alpha \)-mannosidase (-----), \( \beta \)-N-acetylglucosaminidase (----), and \( \beta \)-galactosidase (--). Units indicated are milliunits. Fractions pooled are indicated by the solid bars. \( b \), chromatography of Fraction CM-III (100 mg of protein) on DEAE-cellulose (Whatman DE-32, microgranular). The column, 2.4 x 24 cm, was run at room temperature at a flow rate of 60 to 70 ml per hour; 10-ml fractions were collected. The column was developed initially with 370 ml of 0.020 M NaH\(_2\)PO\(_4\), pH 6.50, and then a concave gradient over 1900 ml (1400-ml mixing chamber, 500-ml reservoir) to 0.30 M NaCl in the same buffer was applied to complete development. Aliquots of 25 \( \mu \)l of the effluent were assayed for \( \alpha \)-fucosidase (---) and \( \beta \)-N-acetylglucosaminidase (----). Units indicated are milliunits. In \( b \) the absorbance at 280 nm is given by (-----). Essentially all of the \( \alpha \)-mannosidase and \( \beta \)-galactosidase emerged with the nonabsorbed fraction. Fractions pooled are indicated by the solid bars.

### Table II

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Treatment</th>
<th>( \alpha )-Fucosidase</th>
<th>( \alpha )-Mannosidase</th>
<th>( \beta )-Glucosaminidase</th>
<th>( \beta )-Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM Fraction I</td>
<td>2 hours, 37(^\circ), pH 3.25</td>
<td>0.007</td>
<td>0.004</td>
<td>0.048</td>
<td>51</td>
</tr>
<tr>
<td>DEAE Fraction I</td>
<td>24 hours, room temperature in 0.005 M cupric acetate, 0.002 M Na(_2)</td>
<td>0.02</td>
<td>2.5</td>
<td>380</td>
<td>5.4</td>
</tr>
<tr>
<td>DEAE Fraction III</td>
<td>15 min, 70(^\circ), pH 6.00</td>
<td>152</td>
<td>0.033</td>
<td>0.72</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*Units are for 20 \( \mu \)l of enzyme solution.
*If the azide is omitted (5 mM copper concentration), the recovery of \( \beta \)-glucosaminidase is about 70\% with greater than 90\% inactivation of the other activities.

also determined over a period of 200 hours under the conditions of glycoprotein or glycopeptide hydrolysis. This experiment was carried out with both the isolated enzyme fractions and with the 35 to 50\% ammonium sulfate fraction; the results for the isolated fractions are given in Fig. 3 and show that all but the mannosidase retain good proportions of their activity with the glucosaminidase the most stable. The results with the 35 to 50\% ammonium sulfate fraction were essentially the same except that the \( \alpha \)-fucosidase retained 80\% of its activity after 200 hours and the \( \beta \)-N-acetylglucosaminidase and \( \beta \)-galactosidase retained 95\%. The instability of the \( \alpha \)-mannosidase is in agreement with the findings of Conchie and Hay (11) and Snaith and Levy (12).

**Effect of Metals and Chelators** The enzymes (35 to 50\% ammonium sulfate fraction) were incubated at room temperature for 20 hours in both acetate and citrate buffers at pH 6 containing various metal ions or chelating agents. The metal concentrations were 5 mM, EDTA was 25 or 50 mM, sodium fluoride and sodium oxalate were 25 mM, and \( \alpha \)-phenanthroline and 8-hydroxyquinoline were 1 or 2 mM.

Salts of heavy metals, Ag\(^{++}\), Hg\(^{++}\), Cu\(^{++}\), Cd\(^{++}\), Co\(^{++}\), Ni\(^{++}\), and Pb\(^{++}\), are generally quite effective inhibitors of the \( \alpha \)-fucosidase and \( \alpha \)-mannosidase in the presence of acetate except for Co\(^{++}\) in the case of the \( \alpha \)-fucosidase. The \( \beta \)-galactosidase and \( \beta \)-N-acetylglucosaminidase are inhibited only by Ag\(^{++}\), Hg\(^{++}\) and Cu\(^{++}\) and the \( \beta \)-N-acetylglucosaminidase is somewhat resistant to inhibition by Cu\(^{++}\). Zinc acetate inhibits the \( \alpha \)-fucosidase greater than 90\%, but has little or no effect on the other enzymes. Aminoff and Furukawa have reported that the \( \alpha \)-(1,2)-fucosidase from *Clostridium perfringens* is likewise inhibited by zinc (2).

No significant inhibition was observed with the chelators either
singly or in combination even if the enzyme preparation was dialyzed against acetate buffer containing 25 mM EDTA. These results, as they concern the α-mannosidase, appear to conflict with the data of Snaith and Levy (12). However, their data are quite compelling, suggesting that the native enzyme binds zinc very tightly but that they may have labilized their enzyme preparation with respect to the zinc by treatment with pyridine or acetone.

Effect of pH on Enzyme Activity—The pH optima, determined with the 35 to 50% ammonium sulfate fraction, were: α-fucosidase, 6.3; α-mannosidase, 4.3; β-N-acetylglucosaminidase, 5.1; and β-galactosidase, 2.8. The data are presented in Fig. 4. Our results are in general accord with those of Levy and McAllan (10) and Conchie and Hay (11) except that the latter authors report a pH optimum of 5.0 for α-mannosidase. The pH optimum of the β-N-acetylglucosaminidase has not been previously reported.

Isolelectric Points—A crude enzyme fraction precipitating between 0 and 50% of ammonium sulfate saturation was subjected to isoelectric focusing by the method of Vesterberg and Svensson (32) with a 110-ml electrofocusing column (LKB Instrument Co., Rockville, Md.). Each activity was separated into two major components except for the α-fucosidase. The isoelectric points found were; α-fucosidase, 6.3; α-mannosidase, 3.9 and 4.2; β-N-acetylglucosaminidase, 4.4 and 8.0; and β-galactosidase, 4.8 and 6.1. The recoveries of activity (total) were 25% for α-fucosidase, 3% for α-mannosidase, 12% for β-N-acetylglucosaminidase, and 75% for β-galactosidase. A control enzyme-ampholine solution stored at room temperature during the electrofocusing run retained full activity of all the enzymes. The low yield of α-mannosidase recovered is probably due to instability at its isoelectric points (see Fig. 2), and the same may be the case for the β-N-acetylglucosaminidase if its major component has an isoelectric point of 8. The other enzymes are quite stable at their isoelectric points. The column profile is shown in Fig. 5.

Physical Properties and Composition of α-α-Fucosidase—The purified enzyme, subjected to high speed sedimentation equilibrium, appeared homogeneous (a plot of log C versus X² gave a straight line). A molecular weight of 216,000 ± 8,000 was calculated. Sedimentation equilibrium in the presence of 6 M guanidine-HCl and 0.5% β-mercaptoethanol also resulted in straight line plots of log C versus X². However, the slope of this plot at a rotor speed of 30,000 rpm was less than at 36,000 rpm. The data indicate the presence of two components under these conditions with molecular weights of 47,000 ± 2,000 and 59,000 ± 2,000. The latter value is probably low as it is an average molecular weight of material containing some of the lower molecular weight component.

Gel electrophoresis of the native material was difficult. As shown in Fig. 6b, the protein did not readily penetrate a 7% polyacrylamide gel at pH 9.5 and may have dissociated in the process. In 3% gels at pH 9.5 no staining was observed, indicating that the protein was not fixed in gels of this porosity. Successful runs were obtained in 5.6% gels at pH 6.6, although an artifact of the
electrophoretic system appeared. After 2 hours of electrophoresis, penetration of the protein was again limited to the top 10 mm of the gel, but after 3 hours results as given in Fig. 6 were consistently found. One protein component was seen, indicated by the arrow, and enzyme activity was found exclusively in the segments of the gel containing this band. The sharp band seen directly below the protein and in the control is an artifact of the system. After runs of 2 hours, enzyme activity also coincided with the protein band at the top of the gel.

The enzyme was subjected to electrophoresis in polyacrylamide gels in the presence of 0.1% SDS. With 20 µg of material, two major bands were seen. Molecular weights of 49,700 and 53,700 were calculated. These results substantiate those found in the ultracentrifuge with regard to the subunit nature of the enzyme. At a higher concentration, 30 µg of material, one broad band was seen with an indicated molecular weight of 59,900. In each gel a very faint band was also observed with a molecular weight of about 130,000. The results are shown in Fig. 6a.

Although the amounts of the fucosidase available were insufficient for detailed chemical studies, the centrifugal and electrophoretic studies indicated that the enzyme preparation was homogeneous or nearly so. Thus, the results of a preliminary analysis of the amino acid and carbohydrate content are shown in Table III. The material is glycoprotein in nature (0.6% carbohydrate) and it is relatively rich in leucine and histidine as compared to many proteins.

Hydrolysis of Glycoprotein and Glycopeptide Oligosaccharides with Rat Epididymal Glycosidases—A variety of substrates are hydrolyzed with the 35 to 50% ammonium sulfate fraction for 72 hours at 37°. The results are given in Table IV. It should be noted that the enzyme was subjected to electrophoresis in the presence of SDS. Electrophoresis was for 7½ hours at 0.5 ma per tube (27 volts) in a 5% acrylamide gel containing 0.1% SDS. One gel (left), with 20 µg of α-fucosidase, shows two major bands with apparent molecular weights of 49,700 and 53,700. The second gel (right), with 30 µg of α-fucosidase shows a single broad band with an apparent molecular weight of 53,900. A faint band (arrow) with a molecular weight of 130,000 was also observed in each gel.

Fig. 6. a, polyacrylamide gel electrophoresis of the purified α-fucosidase in the presence of SDS. Electrophoresis was for 7½ hours at 0.5 ma per tube (27 volts) in a 5% acrylamide gel containing 0.1% SDS. One gel (left), with 20 µg of α-fucosidase, shows two major bands with apparent molecular weights of 49,700 and 53,700. The second gel (right), with 30 µg of α-fucosidase shows a single broad band with an apparent molecular weight of 53,900. A faint band (arrow) with a molecular weight of 130,000 was also observed in each gel. b, polyacrylamide gel electrophoresis of the purified α-fucosidase. Electrophoresis was for 1, 3, and 6 hours (left to right) at 2.5 ma per tube in a 7% acrylamide gel. Gel buffer was 0.126 M Tris-phosphate, pH 9.0; running buffer was 0.012 M sodium glycinate, pH 9.5. Each gel contained 60 µg of protein. c, electrophoresis of the native fucosidase at pH 6.6 for 3 hours at 2 ma per tube in a 5.5% acrylamide gel. Gel buffer was 0.065 M Tris-phosphate, pH 6.6; running buffer was 0.012 M sodium glycinate, pH 6.6. On the left is a control with the enzyme preparation omitted. On the right, 30 µg of enzyme were subjected to electrophoresis. The sharp band in both gels is an artifact (see text); the arrow indicates the single band of protein observed in the sample.

Table III

Amino acid and carbohydrate composition of purified α-fucosidase

The calculation of glucosamine was based on assumed 90% recovery.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per 216,500 mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>110</td>
</tr>
<tr>
<td>Histidine</td>
<td>50</td>
</tr>
<tr>
<td>Arginine</td>
<td>70</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>184</td>
</tr>
<tr>
<td>Threonine</td>
<td>100</td>
</tr>
<tr>
<td>Serine</td>
<td>124</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>204</td>
</tr>
<tr>
<td>Proline</td>
<td>138</td>
</tr>
<tr>
<td>Glycine</td>
<td>146</td>
</tr>
<tr>
<td>Alanine</td>
<td>129</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>34</td>
</tr>
<tr>
<td>Valine</td>
<td>130</td>
</tr>
<tr>
<td>Methionine</td>
<td>58</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>160</td>
</tr>
<tr>
<td>Leucine</td>
<td>80</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>90</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.4</td>
</tr>
<tr>
<td>Fucose</td>
<td>30</td>
</tr>
<tr>
<td>Mannose</td>
<td>10</td>
</tr>
<tr>
<td>Galactose</td>
<td>12</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>Trace</td>
</tr>
<tr>
<td>Galactosamine</td>
<td></td>
</tr>
</tbody>
</table>

1 An artificial band formed 45 min after electrophoresis began in the control and 15 to 30 min later in the sample tubes. Thus the artifact moved further in the controls. It was yellow in color before staining with the protein stain. The fucosidase solution (0.6 mg per ml) contained no trace of yellow color. The artifact was not eliminated by addition of mercaptoethanol (1%), a freshly purchased supply (Eastman) of acrylamide or the substitution of 4% glycerol for the 40% sucrose present in the sample solution when introduced onto the gel. The same reagents at pH 9 to 9.5 do not result in production of the artifact.
noted that as with many similar studies with glycosidases from other sources, release of sugars is not complete even after 72 hours. Most evident is the difference between the glycopeptides and the native glycoproteins in terms of their suitability as substrates. The oligosaccharides of the peptides are hydrolyzed fairly extensively; however, there are interesting differences in the extent of hydrolysis between the various peptides. The Fc peptides are all hydrolyzed to a similar degree, particularly with respect to mannose and N-acetylgalactosamine, thus indicating that their oligosaccharide structures are basically the same. The L peptide from horse immunoglobulin and the LH-β-peptide, which have very different compositions, yield results which are distinct from those of the Fc peptides. It should be noted that the LH-β glycopeptide contains no galactose but does contain 2 moles of N-acetylgalactosamine per mole of peptide, none of which was released.

In contrast, the native glycoproteins are generally quite resistant to glycosidase hydrolysis although galactose was released from human immunoglobulin and about two-thirds of a residue of N-acetylgalactosamine is released from each of the immunoglobulins. Small amounts of mannose and N-acetylgalactosamine were released from native LH, together with a trace of N-acetylgalactosamine. The isolated β chain of LH was not hydrolyzed at all, indicating that the small amount of carbohydrate released from native LH probably originated in the (II chain.

Time studies of the hydrolyses were carried out with the purified α-fucosidase (Fraction DEAE-III) on the LH-β peptide and the horse Fc peptide with the results shown in Fig. 7. In the case of the LH-β peptide no sugar other than fucose was released and removal of this sugar was complete in 200 hours, thus showing that all the fucose is present in a nonreducing terminal position and is α-1 linked. In addition to fucose, N-acetylgalactosamine and galactose were released from the horse Fc peptide. The amounts of these sugars released were surprisingly large considering that these activities, as measured against synthetic substrates, were only 3.8 and 0.25%, respectively, of the fucosidase activity. The N-acetylgalactosamine released represented about one-third of the total (1.5 of 4.0 residues). About two-thirds of the galactose was also released; no release of mannose was observed. Again, release of fucose was complete in 100 hours. Work by Rothfus and Smith (33) is also consistent with the view that fucose and galactose are terminal residues in the homologous human peptide. It is evident that, while the α-fucosidase preparation is sufficiently free of other glycosidases to be useful in cases such as the LH-β peptide, the small amounts of contaminating β-N-acetylgalactosaminidase (3.8% based on activity against

---

**Table IV**

Sugars released from some glycoproteins and glycopeptides by glycosidases

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percentage of carbohydrate released</th>
<th>Amount of carbohydrate released per mole of substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Faucose</td>
<td>Mannose</td>
</tr>
<tr>
<td>Horse IgG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human IgG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LH-β chain</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Horse IgG Fc peptide</td>
<td>52</td>
<td>53</td>
</tr>
<tr>
<td>Human IgG Fc peptide</td>
<td>92</td>
<td>63</td>
</tr>
<tr>
<td>Rabbit IgG Fc peptide</td>
<td>52</td>
<td>55</td>
</tr>
<tr>
<td>Horse IgG L peptide</td>
<td>54</td>
<td>13</td>
</tr>
<tr>
<td>LH-β peptic glycopeptidesb</td>
<td>66</td>
<td>6</td>
</tr>
</tbody>
</table>

* The carbohydrate moiety of this peptide contains 1 residue of sialic acid.

b This was a mixture containing a tetrapeptide, a hexapeptide, and a tetradecapeptide. All are from the same sequence and have the same carboxyl terminus.
or-fucosidase (0.6 mg per ml, in 0.1 M ammonium acetate buffer, pH 8.0) is unstable outside the pH range 5 to 7. In practice it was necessary to restrict the pH range for column chromatography to 5.5 to 6.5 for the best recovery of the fucosidase. Because several of the enzymes do not bind strongly to cellulose ion exchangers under such conditions of ionic strength and near-neutral pH, the performance of the columns is fairly sensitive to alterations of conditions, although, after suitable conditions were found, the final chromatographic fractionation was simple and reproducible. Finally, except for the α-mannosidase, which is the least stable of these enzymes, much of the contaminating glycosidic activities in the individual glycosidase preparations can be inactivated. However, none of these procedures is completely effective and further work on inactivation or improvement of the chromatography is required.

The purified α-fucosidase, while still contaminated with small amounts of other glycosidase activities, appeared to be essentially homogeneous by the criterion of gel electrophoresis at pH 6.6 (as well as by the less rigorous centrifugal criterion). Only a single protein band was observed and the fucosidase activity coincided with the band. The material dissociates in the presence of 6 M guanidine or sodium dodecyl sulfate and the ultra centrifugal and electrophoretic measurements suggest that the native enzyme is a tetramer. Further studies are needed to establish conclusively that the subunits consist of two pairs of unlike chains with molecular weights of 47,000 and 69,000 as indicated by the data. The analyses of composition show that the enzyme is glycoprotein containing 0.9% carbohydrate. Other glycosidases that have been chemically characterized include β-galactosidase from E. coli (35) and the β-N-acetylglucosaminidase from Aspergillus oryzae (36); the latter is also a glycoprotein. Physical and chemical characterization of the epididymal mannosidase (12) and of the bacterial fucosidases (1, 2) have not yet been reported.

The fucosidase is primarily of interest in terms of its ability to hydrolyze glycoprotein oligosaccharides. The obvious differences in the degree of hydrolysis of peptides as opposed to that of native glycoproteins probably result from steric hindrance, with smaller substrates more easily hydrolyzed. The obvious differences observed between the two peptides in the amounts of mannose and N-acetylglucosamine released suggest significant structural differences between them, although it is possible that differences in amino acid sequence might have affected the hydrolysis.

The time studies of the hydrolyses of the LH-β Fc peptide and horse Fc peptide demonstrate that the fucose is completely removed from both of these dissimilar peptides by the purified α-fucosidase and so the fucose in these structures must be α-1 linked. It was surprising, however, that the traces of β-N-acetylglucosaminidase and β-galactosidase activity exhibited against the synthetic substrates and β galactosidase (0.25%) in the preparation also cause release of appreciable quantities of these two monosaccharides in other cases. Further attempts should be made to inactivate the residual contaminations. Hydrolyses with the α-fucosidase after heating at 70° were not carried out because this preparation was still contaminated with 0.5% β-N-acetylglucosaminidase and 0.2% β-galactosidase.

The data concern two major aspects of the α-1-fucosidase from rat epididymis, its physical and chemical properties and its suitability for use in studies on the structures of the oligosaccharide moieties of glycoproteins. The epididymis has been shown to contain a number of different glycosidase activities, principally α-fucosidase, α-mannosidase, β-N-acetylglucosaminidase, and β-galactosidase. It also contains very low levels (1 to 2% or less of the activity of the four principal epididymal glycosidases) of α- and β-glucosidase, α-galactosidase, and β-mannosidase. It is lacking β-fucosidase, β-xylanase, and may have a trace of α-N-acetylglucosaminidase activity (10, 13). Of these enzymes, the α-mannosidase is the only one which has previously been obtained free of other glycosidase activities (12). The work described herein is the first purification of a mammalian fucosidase which, of the epididymal enzymes, appears to be the most useful as an addition to the glycosidases, from other sources, which are valuable in structural studies. The nonmammalian α-fucosidases which have been isolated are characterized as 1,2-α-L-fucosidases and seem to require a fucosyl-galactose bond (1, 2); thus their specificity is very narrow. The epididymal fucosidase has a broader specificity as it is active against both synthetic substrate (10) and against a variety of glycoprotein oligosaccharides (18, 19). Further, it is active against an oligosaccharide (from LH-β) which contains no galactose.

In the course of purifying the α-fucosidase, the separation and partial purification of the other major epididymal glycosidases also resulted; the latter may also be useful after further purification. All are active against glycopeptide substrates (18, 19, 34). However, with the exception of the α-fucosidase, which has a specificity different from the other α-fucosidases described, enzymes which are probably equally effective have recently become available from other sources in apparently pure form (3-9). An important observation made in this study is that the epididymides, in 30% glycerol at −20°, can be stored for at least 2 years without significant losses of enzyme activity. Further, the enzymes, either in crude extracts or as the purified fractions, may be stored in solution at 4° for 3 months with less than a 10% loss in any activity.4 The preparation of the crude extract is quite straightforward, involving only homogenization and incubation of the homogenate at 37° to release the enzymes as described by Levvy and McAllan (10), followed by a heat step and fractionation with ammonium sulfate. Large amounts of inert protein are removed by the heat treatment. The crude extract is then subjected to chromatography on CM- and DEAE-celluloses. There were two major difficulties in developing the chromatographic procedures; first, the enzymes are unstable below an ionic strength of about 0.05 and, secondly, the α-fucosidase is unstable outside the pH range 5 to 7. In practice it was necessary to restrict the pH range for column chromatography to 5.5 to 6.5 for the best recovery of the fucosidase. Because several of the enzymes do not bind strongly to cellulose ion exchangers under such conditions of ionic strength and near-neutral pH, the performance of the columns is fairly sensitive to alterations of conditions, although, after suitable conditions were found, the final chromatographic fractionation was simple and reproducible. Finally, except for the α-mannosidase, which is the least stable of these enzymes, much of the contaminating glycosidic activities in the individual glycosidase preparations can be inactivated. However, none of these procedures is completely effective and further work on inactivation or improvement of the chromatography is required.

The purified α-fucosidase, while still contaminated with small amounts of other glycosidase activities, appeared to be essentially homogeneous by the criterion of gel electrophoresis at pH 6.6 (as well as by the less rigorous centrifugal criterion). Only a single protein band was observed and the fucosidase activity coincided with the band. The material dissociates in the presence of 6 M guanidine or sodium dodecyl sulfate and the ultra centrifugal and electrophoretic measurements suggest that the native enzyme is a tetramer. Further studies are needed to establish conclusively that the subunits consist of two pairs of unlike chains with molecular weights of 47,000 and 69,000 as indicated by the data. The analyses of composition show that the enzyme is glycoprotein containing 0.9% carbohydrate. Other glycosidases that have been chemically characterized include β-galactosidase from E. coli (35) and the β-N-acetylglucosaminidase from Aspergillus oryzae (36); the latter is also a glycoprotein. Physical and chemical characterization of the epididymal mannosidase (12) and of the bacterial fucosidases (1, 2) have not yet been reported.

The fucosidase is primarily of interest in terms of its ability to hydrolyze glycoprotein oligosaccharides. The obvious differences in the degree of hydrolysis of peptides as opposed to that of native glycoproteins probably result from steric hindrance, with smaller substrates more easily hydrolyzed. The obvious differences observed between the two peptides in the amounts of mannose and N-acetylglucosamine released suggest significant structural differences between them, although it is possible that differences in amino acid sequence might have affected the hydrolysis.

The time studies of the hydrolyses of the LH-β Fc and horse Fc peptide demonstrate that the fucose is completely removed from both of these dissimilar peptides by the purified α-fucosidase and so the fucose in these structures must be α-1 linked. It was surprising, however, that the traces of β-N-acetylglucosaminidase and β-galactosidase activity exhibited against

4 A paper reporting on the structures of glycopeptides from human myeloma proteins has just appeared (38). A partially purified preparation of the fucosidase was used in conjunction with other glycosidases.
the synthetic substrates were manifested so markedly in the hydrolysis of the horse Fc peptide. This observation clearly delineates the hazards of extrapolation of activity against synthetic substrates to the interpretation of oligosaccharide structures unless kinetic studies and quantitative identification of each sugar released are made. This can be done readily by the method described herein, in which the released sugars are separated from the other components of the digestion mixture by ultrafiltration and ion exchange chromatography. Other approaches are to use gel filtration (34, 39) or adsorption of the sugars on activated charcoal (1), although, in our hands, difficulties were encountered in achieving the required separations.

With LH-2 peptide, fucose was the only sugar released, thus all must be present as a nonreducing terminus.

Acknowledgments—We express appreciation to Miss Tiu Reino for helpful assistance, to Mr. D. M. Brown for the ultracentrifugal analyses, and to Dr. Derek Chignell for some of the gel electrophoresis.

REFERENCES

Purification and Properties of an α-L-Fucosidase from Rat Epididymis
Robert B. Carlsen and John G. Pierce


Access the most updated version of this article at http://www.jbc.org/content/247/1/23

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/247/1/23.full.html#ref-list-1