Purification and Characterization of \( \alpha\)-Mannosidase from Rat Liver Golgi Membranes

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Rat liver contains three \( \alpha\)-mannosidases occurring in different intracellular fractions. The present paper reports the isolation of the mannosidase of Golgi membranes, in which the enzyme is a distinctive glycosidase component. The Golgi mannosidase was extracted with detergent and purified to apparent homogeneity, all solutions requiring the presence of detergent (0.1% Triton X-100) to maintain the enzyme in soluble form. In molecular weight determinations gel chromatography on Sephadex G-200 yielded a value of 295,000, whereas sucrose density gradient centrifugation gave a value of 110,000. Under dissociating conditions, polyacrylamide gel electrophoresis showed two bands, corresponding to molecular weights of 75,000 to 80,000 and 145,000 to 150,000. It is tentatively concluded that the mannosidase is a tetrameric protein of approximately 300,000 molecular weight, and that the dimeric form is relatively stable. The pH optimum is 5.5; the isoelectric point is 5.8.

Several \( \alpha\)-mannosidases (EC 3.2.1.24) occur in mammalian tissues. Liver lysosomes contain "acid" \( \alpha\)-mannosidase long considered to be involved in glycopolymer breakdown. Marked deficiency of the lysosomal enzyme activity causes the storage disease mannosidosis in man (1, 2) and cattle (3). In 1971 Marsh and Gourlay (4) discovered a cytosolic or "neutral" \( \alpha\)-mannosidase in rat liver; its purification and characterization have recently been reported by Shoup and Touster (5).

The third type of rat liver \( \alpha\)-mannosidase, encountered first by Dewald and Touster (6) in Golgi membranes, is the subject of the present report. Interest in this enzyme rests on the generally accepted role of the Golgi apparatus in glycoprotein biosynthesis (7), on the suggested (8) but unproven (9) role of this organelle in lysosome formation, and on the fact that glycosidases are not generally present in Golgi membranes (6).

**EXPERIMENTAL PROCEDURES**

Experimental procedures are presented in the miniprint supplement to this communication.¹

**RESULTS**

Extraction of \( \alpha\)-Mannosidase from Golgi Membranes—Golgi membranes were suspended in Tris/HCl buffer (50 mM, pH 7.2) containing 5 mM MgCl\(_2\) and 0.3% Triton X-100 (4 to 4.5 mg of protein/ml of buffer) by manual homogenization in a glass homogenizer with a Teflon pestle. The suspension was kept on ice for 15 min and then centrifuged at 50,000 rpm (165,000 \( \times \) g) for 60 min. The supernatant solution was carefully removed, by aspiration, from the pellet and from the 0.5 ml of loosely packed membranes above the pellet. The pellet and membranes were resuspended in the same volume of buffer, homogenized and centrifuged at 50,000 rpm for 60 min as above. This process was repeated four more times. The pellet remaining after the sixth extraction was suspended in the Tris/HCl/Triton X-100 buffer. \( \alpha\)-Mannosidase and protein were assayed in the original Golgi suspension, the six extracts, and the final suspension. The first extract contained 42% of the total protein and 8 to 10% of the \( \alpha\)-mannosidase. The second through sixth extracts contained about 70% of the Golgi mannosidase activity but only 46% of the protein and that the dimeric form is relatively stable. The pH optimum is 5.5; the isoelectric point is 5.8.

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¹ Experimental procedures are presented in a miniprint format immediately following this paper (Figs. 3, 6, 7, and 11 and Table I are found on p. 3233). For the convenience of those who prefer to obtain the supplementary material in the form of 8 pages of full size photocopies, it is available as JBC Document Number 76M-1294. Orders should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.20 per set of photocopies.

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0.3% Triton X-100. In order to further study this phenomenon, Golgi membranes washed with 0.4 M NaCl were suspended in Tris/HCl buffer (50 mM, pH 7.2) containing 5 mM MgCl₂ and 0.3% Triton X-100 at protein concentrations varying from 2.0 to 7.3 mg/ml and then repeatedly extracted as described above for extraction of α-D-mannosidases. The solubilization of protein and α-mannosidase activity by the Triton X-100 solution is presented in Fig. 1. When the initial concentration of protein is low, most of the activity is extracted in the first or second extract. As the initial protein concentration increases, the majority of the activity is solubilized in the third and fourth extracts. However, regardless of the initial concentration of protein, the specific activity in the supernatant solutions increases as the number of extracts increases. By using a high protein concentration we were able to maximize the yield of enzyme recovered, as well as obtain a 50% increase in specific activity.

The second through sixth extracts were combined and diluted to 0.1% (v/v) Triton X-100 with 50 mM Tris/HCl buffer, pH 7.2, containing 5 mM MgCl₂. This diluted enzyme was applied to a DEAE-cellulose column (1 x 16 cm, Whatman DE52) equilibrated with 50 mM Tris/HCl buffer, pH 7.2, containing 5 mM MgCl₂ and 0.1% Triton X-100 and washed with 30 ml of this buffer. Sixty to 70% of the α-mannosidase activity applied passed directly into the effluent without being retarded and was found to be increased 3- to 4-fold in specific activity. The remaining 10 to 20% of the activity, which was not further used in this enzyme purification, was adsorbed to the column and could be eluted with 0.5 M NaCl in 50 mM Tris/HCl buffer (pH 7.2) containing 0.1% Triton X-100.

The effluent and washing from the DEAE-cellulose column (150 to 200 ml) were pumped onto a column (1 x 17 cm) of fine mesh cellulose/phosphate (Sigma) equilibrated with 50 mM Tris/HCl buffer, pH 7.2, containing 0.1% Triton X-100 and 5 mM MgCl₂. The column was washed with 200 ml of the same buffer and then with a 250-ml linear NaCl gradient (0 to 0.5 M) in the same buffer to elute the enzyme. Five-milliliter fractions were collected at a flow rate of 40 ml/h. The α-mannosidase activity appeared in fractions 7 to 16; it was purified 3- to 4-fold, with a 30 to 40% yield of activity.

The enzyme activity was pooled and pumped onto a hydroxylapatite column (1.5 x 6 cm) equilibrated with 40 mM potassium phosphate buffer, pH 7.2, containing 0.1% Triton X-100, at a flow rate of 30 ml/h. The column was washed with 150 to 200 ml of the same buffer followed by elution with 30 ml of 0.1 M potassium phosphate buffer, pH 7.2, containing 0.1% Triton X-100. This procedure purified the enzyme 4- to 5-fold with a 42% yield of activity. The enzyme solution was concentrated to 3 ml with an Amicon concentrator equipped with an XM-50 membrane. A summary of the purification procedure for α-mannosidase is presented in Table III.

Purified Golgi α-mannosidase was very stable when stored either at 5° or frozen in the presence of potassium phosphate buffer (0.1 M, pH 7.2) and Triton X-100. After 30 days at 5° about 80% of the initial activity remained. The enzyme can be kept frozen for at least 2 months without any loss of activity. Freezing and thawing has no effect upon purified α-mannosidase; after eight cycles of freezing and thawing, nearly all the activity was recovered in the supernatant.

**Attempts to Remove Detergent from Purified Enzyme** – The purified concentrated enzyme described above, which contains about 1% Triton X-100 as assayed by the method of Holloway (18), is completely soluble when centrifuged at 50,000 rpm for 60 min. The Golgi α-mannosidase requires detergent to remain solubilized, as is indicated by the fact that the enzyme becomes insoluble and precipitates on the column when it is centrifuged.

### Table II

| Fraction          | Protein Recovery | Manelli Recovery |
|-------------------|------------------|------------------|
| Original suspension | 50.0 mg | 100.0% | 1.98% |
| Extract 1         | 24.9 mg | 42.2% | 0.185% |
| Extract 2         | 15.9 mg | 26.9% | 0.613% |
| Extract 3         | 9.0 mg  | 15.4% | 0.448% |
| Extract 4         | 3.5 mg  | 6.1%  | 0.216% |
| Extract 5         | 0.5 mg  | 0.9%  | 0.067% |
| Extract 6         | 0.2 mg  | 0.3%  | 0.034% |
| Final pellet suspension | 5.5 mg | 9.5% | 0.296% |

*The protein values given are approximate due to the fact that the relatively large volumes which were required for the assay of protein contained concentrations of Triton X-100 which interfere with the Miller protein assay.*

![Fig. 1.](http://www.jbc.org/)

Fig. 1. Extraction of proteins (A) and α-D-mannosidase (B) from the Golgi membranes with Triton X-100. Fresh Golgi membranes were suspended in 50 mM Tris/HCl buffer, pH 7.2, containing 5 mM MgCl₂ by manual homogenization in a glass homogenizer with a Teflon pestle. The suspension was kept on ice for 15 min and then centrifuged at 50,000 rpm (165,000 × g) for 60 min. The supernatant (Extract 1) was removed by aspiration, and the pellet along with loose membranes was resuspended in an equal volume of the above buffer, homogenized, and centrifuged as above. This process was repeated twice more. Protein and enzyme were assayed in the original Golgi suspension and four extracts as given in the miniprint supplement. Protein (A) and enzyme activity (B) of the original suspension are expressed as per cent extracted. The relative specific activity (per cent activity extracted/per cent protein extracted) is presented in C. Three different concentrations of Golgi membranes were tested in this experiment; the concentration used (expressed as protein) were 1.95 mg/ml ( ), 3.8 mg/ml ( ), and 7.1 mg/ml ( ).
chromatographed on hydroxylapatite or Sephadex G-200 in the absence of detergent. Most of the activity can be recovered from both of these columns by the addition of 0.15% Triton X-100 to the appropriate elution buffer. When the enzyme is centrifuged through 5 to 20% sucrose gradients in the absence of detergents at 50,000 rpm for 8 h, the activity moved to the bottom of the gradient, whereas in the presence of 0.1% Triton X-100 the enzyme moves only one-third that distance.

Attempts to remove the detergent with Bio-Beads SM-2 (18) without causing precipitation of the enzyme were unsuccessful, as is shown by the following experiment. Purified enzyme solution (1.5 ml, 0.05 units/ml) in 0.1 M potassium phosphate buffer, pH 7.2, and 1% Triton X-100 were mixed with Bio-Beads (washed according to Holloway (18)) (0.4 g/ml) in a glass conical tube. The sample was allowed to stand on ice for 2 h with occasional shaking. Duplicate aliquots were removed at various time intervals for determination of Triton X-100 and α-mannosidase. After 2 h the detergent concentration had been reduced to 0.07%, but there was no diminution in α-mannosidase activity. However, centrifugation of the suspension at 50,000 rpm (165,000 g) for 60 min sedimented 75 to 80% of the activity with the Bio-Beads. Mixing the pellet with an equal volume of 0.5% Triton X-100, 0.1 M potassium phosphate buffer, pH 7.2, and then incubating for 5 min at 3°C restored the enzyme to an unsedimentable state. Another experiment employing a Bio-Bead column gave similar results. In brief, a Triton X-100 concentration of at least 0.1% is required to keep the enzyme in solution.

Electrophoresis of Golgi α-Mannosidase—Purified α-mannosidase was examined by analytical gel electrophoresis in 7% polyacrylamide gels according to Davis (19). When low amounts of protein (5 μg) are applied to gels containing Triton and deoxycholate, and the enzyme moves as a single band as indicated by both protein and activity stains (Fig. 2A). Increasing the deoxycholate concentration increases the mobility of enzyme (Fig. 2F). When larger quantities (15 μg) of protein are applied, two bands appear, both of which stain for protein and activity (Fig. 2, C and D). If Triton is the only detergent used, two bands also appear (Fig. 2B), and both stain for protein and activity. When electrophoresis is performed in the presence of Triton X-100 under the acidic conditions used by Reisfeld et al. (20), the enzyme remains at the origin and no other bands of protein are found.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was used to estimate the subunit molecular weight of the enzyme. The method of Laemmli, as modified by Bock and Fleischer (25) by inclusion of 4 mM urea and 1 mM EDTA in the gel, yielded two bands corresponding to apparent subunit molecular weights of 150,000 and 75,000 respectively. If the enzyme was carboxymethylated by the procedure of Pitt-Rivers and Impiombato (27) and then electrophoresed in sodium dodecyl sulfate by the procedure of Weber and Osborn (26), two bands appeared with apparent molecular weights of 145,000 and 80,000 (Fig. 3).

Approximately 35 μg of purified enzyme was electrophoresed (Davis system) and stained for carbohydrate by the periodic acid-Schiff procedure of Kapitany and Zebroski (22). The positive result suggested that the enzyme is a glycoprotein. When the same amount of protein was stained for lipid and electrophoresed, no lipid bands were observed, although positive results were obtained for serum and crude Golgi membrane extract.

Molecular Weight Determinations—The molecular weight of α-mannosidase as determined by sucrose density centrifugation was 110,000 when referred to catalase as marker and 120,000 in reference to glucose-6-phosphate dehydrogenase (Fig. 4). Only one symmetrical peak of enzyme activity was obtained when either 0.1 or 1% Triton X-100 was included in the gradient. As mentioned earlier, when there was no Triton X-100 in the sucrose gradient the enzyme activity was found at the bottom of the gradient.

Molecular sieve chromatography gave a single peak of enzyme activity corresponding to an apparent molecular weight of 256,000 (Fig. 5). The molecular weight value was the same in the presence of either 0.1 or 1% Triton X-100 and was independent of the concentration of sucrose present (0.5, 10% w/v).

Isoelectric Point—Isoelectric focusing of the purified Golgi α-mannosidase in a pH 3 to 10 gradient indicated that the enzyme has an isoelectric point of approximately 5.8 (Fig. 6). The yield of enzyme activity in this experiment was about 85%. Partially purified enzyme (0.7 unit/mg of protein) showed a similar isoelectric point. There was only one symmetrical band of α-mannosidase activity in these experiments.

Activation Energy and Heat Stability—The activation energy of the Golgi α-mannosidase calculated from an Arrhenius plot was 11,000 cal mol⁻¹ (Fig. 7). The enzyme is stable until approximately 40°C, when it becomes very unstable as the temperature is increased further (Fig. 7). A study of the stability of the enzyme at either 37 or 45°C in the presence of various compounds indicated that both Zn²⁺ and high concentrations

| Fraction              | Protein | Enzyme activity | Specific activity | Yield (%) | Purification fold |
|-----------------------|---------|-----------------|------------------|-----------|------------------|
| Golgi-rich fraction   | 56.9    | 1.82            | 0.032            | 100       | 1                |
| Soluble fraction      | 26.6    | 1.38            | 0.052            | 76        | 1.6              |
| DEAE-cellulose        | 3.0     | 1.02            | 0.34             | 56        | 8.2              |
| Cellulose phosphate   | 0.9     | 0.607           | 0.67             | 33        | 21               |
| Hydroxylapatite       | 0.08    | 0.249           | 3.1              | 14        | 97               |

Fig. 2. Electrophoresis of α-mannosidase in polyacrylamide gels. Purified Golgi α-mannosidase was electrophoresed by the method of Davis et al. (19) under the following conditions: A, 0.2% Triton X-100, 0.05% deoxycholate, and 5 μg of protein; B, 0.2% Triton X-100 and 5 μg of protein; C and D, 0.2% Triton X-100, 0.05% deoxycholate, and 15 μg of protein; E, 0.2% Triton X-100, 0.2% deoxycholate, and 5 μg of protein; F, 0.2% Triton X-100 with electrophoresis according to Reisfeld et al. (20). Gel D was stained for α-mannosidase activity, and the other gels were stained for protein.

Table III

| Fraction                          | Protein | Enzyme activity | Specific activity | Yield (%) | Purification fold |
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| Hydroxylapatite                   | 0.08    | 0.249           | 3.1              | 14        | 97               |
Fig. 4. Sucrose density gradient centrifugation of purified Golgi α-D-mannosidase. Mannosidase (M) (0.1 unit of 97-fold purified enzyme) was centrifuged at 50,000 rpm for 6.5 h in a 5 to 20% sucrose gradient containing 0.1% Triton X-100 and 50 mM Tris, pH 7.2. Beef liver catalase (C) and glucose-6-phosphate dehydrogenase (G) from Leuconostoc mesenteroides were used as standards. The ordinate is based on arbitrary values which are proportional to the activities found for each enzyme. Volume of gradient, 5.0 ml; volume of fractions, 0.1 ml.

Fig. 5. Molecular weight determination of purified Golgi α-D-mannosidase by molecular sieve chromatography on a column (100 x 2.5 cm) of Sephadex G-200. β-Galactosidase (1), β-D-glucuronidase (2), glucose-6-phosphate dehydrogenase (3), alcohol dehydrogenase (4), horseradish peroxidase (5), and mannosidase (arrow) were applied to the column and eluted as described in the miniprint supplement. Flow rate, 10 ml/h; volume of fractions, 2.5 ml.

Fig. 8. Stability of purified Golgi α-D-mannosidase. Enzyme (0.2 μg/0.1 ml final volume) was preincubated for various times in 0.1 M sodium acetate buffer, pH 5.5, containing 0.1% Triton X-100 at either 37° (A) or 45° (B) in the presence of 1 mM ZnCl₂ (Δ), 250 mg of bovine serum albumin (○), or no added compounds (■). Enzyme activity at the initiation of the experiment is expressed as 100%. ZnCl₂ (1 mM) and bovine serum albumin (250 mg) were present during the assays of all samples.

Fig. 9. Stability of purified Golgi α-D-mannosidase as a function of pH. Enzyme (1.2 μg) in 0.1 M of buffer and 0.1% Triton X-100 in a total volume of 0.1 ml was preincubated for 60 min at 37° at varying pH. Aliquots containing 0.2 μg of enzyme protein were assayed with 3 mM substrate at pH 5.5 as described in the miniprint supplement. ○, control; ○, preincubation in the presence of 1 mM ZnCl₂. Initial enzyme activity of control was 1.46 milliunits; in the presence of 1 mM ZnCl₂, the enzyme activity was 1.87 milliunits. Sodium acetate buffer was used for pH 4 to 6 and Tris/HCl buffer was used in the pH 7.0 to 9.0 range.

The stability of α-D-mannosidase at 37° with respect to pH is presented in Fig. 9. At acid pH the enzyme is unstable at acid pH and its stability increases as the pH increases. At acid pH Zn⁺² is the more effective (Fig. 8).

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Effector of Mannosidase Activity—Mg⁺², Mn⁺², and Na⁺ had no effect on enzyme activity. Cu⁺² and Fe⁺² are potent inhibitors, whereas Zn⁺², Co⁺², mercaptoethanol, and dithiothreitol are activators (Fig. 12). That the latter agents are indeed activators, rather than stabilizers, is indicated by the fact that the enzymatic reaction in their absence and in their presence is first order with respect to time during the entire reaction period. In fact, the substrate completely stabilizes the enzyme under assay conditions. p-Chloromercuriphenylsulfonylic acid inhibits the enzyme approximately 80% at a concentration of 1 mM. Bovine serum albumin (125 μg/ml) "activates" the enzyme approximately 35%. Ethylene diaminetetraacetic acid (1 mM), 1,10-phenanthroline (0.1 mM), and α,α'-dipyridyl (1 mM) have no effect on enzyme activity. Mannose (250 mM) inhibits the enzyme approximately 30%, fructose (250 mM) stimulates activity 15 to 20%, and glucose (250 mM) and galactose (250 mM) are without effect.

Fig. 10 illustrates the behavior of the purified Golgi α-D-mannosidase as a function of pH over the range of 4.0 to 6.0. The maximum activity was observed at pH 5.5, in agreement with the value reported by Dewald and Touster (6) for crude enzyme in Golgi-rich fraction.

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A number of nucleotides, including AMP, ADP, ATP, GMP,
always found after the first extraction. These data suggest that the concentration of the p-nitrophenyl a-D-mannopyranoside was varied.

**DISCUSSION**

The a-D-mannosidase isolated from the Golgi membranes appears to have a number of properties which clearly differentiate it from the a-D-mannosidases isolated from the lysosomal and soluble fractions of the cell, as well as from a-D-mannosidases which have been characterized from other sources, both animal and microbial.

The pattern of extraction of the mannosidase from the membranes is of considerable interest. We previously used deoxycholate to extract the enzyme because one extraction with Triton X-100 solubilized only a small portion of a-D-mannosidase activity (6). In this study, we have found that repeated extraction with Triton X-100 solubilizes most of the enzyme activity (Fig. 1). This pattern of enzyme release is dependent on protein concentration, although the highest specific activity is always found after the first extraction. These data suggest that the a-mannosidase is not accessible until protein which binds, or covers, the enzyme is removed.

The enzyme requires detergent to remain solubilized during the entire purification procedure. It was purified approximately 5000-fold over the crude cellular homogenate (100-fold over the purified Golgi membranes) and appears to be homogeneous, as indicated by disc gel electrophoresis at pH 8.8 and 5.0. However, when a large amount of protein was placed on the electrophoretic gels, two bands of activity and protein appeared, one of which was always found at or near the origin. We believe the second, more slowly moving form is an artifact resulting from hydrophobic interactions of proteins highly concentrated at the top of the polyacrylamide gel, perhaps due to insufficient Triton X-100.

Molecular weight values differed greatly, depending upon the method employed. Sucrose density centrifugation and molecular sieve chromatography gave values of 110,000 and 295,000, respectively. Molecular sieve chromatography was performed at various sucrose and Triton X-100 concentrations to investigate the possibility that the lower value obtained by sucrose density gradient centrifugation was a result of subunit formation induced by these agents. However, all these experiments yielded only one peak of enzyme activity which corresponded to a molecular weight of approximately 295,000. Because of complexities in the use of sucrose density centrifugation in the presence of detergent, as discussed by Tanford et al. (31), the molecular weight of 295,000 obtained by molecular sieve chromatography is favored over the much lower value obtained by sucrose density gradient centrifugation. Support for the former value was obtained from subunit molecular weight studies.

The subunit molecular weight of the mannosidase was determined in a number of different gel concentrations and electrophoretic systems, as suggested by Segrest and Jackson (32). All conditions yielded two protein bands, corresponding to molecular weights of 150,000 and 75,000, respectively. Since no evidence was found for any contaminant in the highly purified enzyme, we conclude that the subunit molecular weight is approximately 75,000 and that the slower form is a relatively stable dimer with a molecular weight of 150,000.

a-D-Mannosidase apparently does not require any ions for activity, although it is activated by Co^{2+} and Zn^{2+}. It is possible, however, that catalytically important cations are so tightly bound that ethylenediaminetetraacetic acid and the other chelating agents tested cannot remove them from the enzyme.

The Golgi a-mannosidase is activated by the sulfhydryl reagents, mercaptoethanol and dithiothreitol, and is inhibited by p-chloromercuriphenylsulfonic acid. The activation by ATP and by CMP is of possible physiological significance because these nucleotides were effective at 1 mM concentrations. The stimulatory effect of bovine serum albumin may represent a true activation, rather than the stabilization usually attributed to this agent, because the reaction kinetics are linear during the assay even in the absence of albumin.

The physiological role of the Golgi a-mannosidase is unknown. Its properties are very different from those of the cytosolic a-mannosidase of rat liver (5) and from the lysosomal enzyme as well. Substrate specificity studies in progress may provide insights into possible biological functions. It is of interest that the Golgi mannosidase is present in normal human plasma (33) and is the main mannosidase activity demonstrable in bovine mannosidosis plasma (34).

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