Purification and Characterization of Goat Lysosomal \( \beta \)-Mannosidase Using Monoclonal and Polyclonal Antibodies*

(Received for publication, October 3, 1991)

Bryce L. Sopher, Christine E. Traviss, Kevin T. Cavanagh, Margaret Z. Jones, and Karen H. Friderici†

From the Department of Pathology, Michigan State University, East Lansing, Michigan 48824

Goat \( \beta \)-mannosidase was purified 120,000-fold in 26% yield from kidney using concanavalin A-Sepharose chromatography followed by immunoaffinity and cation-exchange chromatography. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining, the purfied enzyme preparation consists of 90- and 100-kDa peptides. Both these peptides react with anti-\( \beta \)-mannosidase monoclonal antibodies and produce similar electrophoretic peptide patterns when subjected to limited proteolysis. Deglycosylation reduces the size of the 90- and 100-kDa peptides to 86 and 91 kDa, respectively. Goat kidney tissues lacking \( \beta \)-mannosidase activity, acquired from animals affected with \( \beta \)-mannosidosis, do not contain detectable quantities of the 90- and 100-kDa peptides as judged by monoclonal antibody reactivity. We postulate that the 90- and 100-kDa peptides represent two related forms of \( \beta \)-mannosidase.

\( \beta \)-Mannosidase has been partially purified and incompletely characterized from several mammalian sources (22-29). Molecular mass estimated by gel filtration range from 80 to 120 kDa. The reported extent of purification as judged by the number of bands observed when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is low, except for guinea pig liver where a major peptide of 110 kDa was demonstrated (30).

We have undertaken the purification and characterization of lysosomal \( \beta \)-mannosidase from normal goat kidney to make possible the biochemical, immunological, and structural characterization of the enzyme, its corresponding gene(s), and the mutations which give rise to \( \beta \)-mannosidosis. These studies may lead to insights regarding the myelin deficits in ruminants, the clinical heterogeneity in humans, and possibly the biochemical basis of a combined deficiency of \( \beta \)-mannosidase and hepatic sulfamidase described by Wenger and colleagues (9). This is the first report of a \( \beta \)-mannosidase purification procedure that yielded highly purified peptides which are modified or absent in tissue from animals affected with \( \beta \)-mannosidosis. This work builds upon preliminary results presented previously (29).

**EXPERIMENTAL PROCEDURES**

**Materials**—Kidneys from mature goats were obtained from M Gross Abattoir in Toronto, Canada. \( \beta \)-Mannosidosis affected and matched control tissues were acquired from our goat colony housed on the Michigan State University campus. All tissues were stored at -20 °C or -80 °C until needed. Concanavalin A-Sepharose 4B (ConA-Sepharose), 4-methylumbelliferyl \( \beta \)-D-mannopyranoside, methyl-a-glucoside, Staphylococcus aureus V8 protease, incomplete Freund’s adjuvant, and Coomassie Blue dye were from Sigma. Leupeptin, pepstatin, protein A-agarose, N-glycosidase F, and endoglycosidase H were from Boehringer Mannheim Biochemicals. Affi-Gel HZ beads and all electrophoresis reagents were from Bio-Rad. ORIGM was from IGM Inc., Rockville, MD. Immobilon was from Millipore Corp., Bedford, MA. RIBI adjuvant was from RIBI Immunological Research Inc., Hamilton, MO. Sodium hypoxanthine, aminopterin, and thymidine and Dulbecco’s modified Eagle’s medium were from Sigma. Bovine fetal calf serum was from HyClone laboratories, Logan, UT.

**Enzyme Assay**—\( \beta \)-Mannosidase activity was assayed at pH 5.0 with 4-methylumbelliferyl \( \beta \)-D-mannopyranoside as described previ-
from Normal and Affected Goat Kidney Tissue—Equivalent amounts (3). One unit of enzyme activity was defined as that amount of enzyme which hydrolyzed 1 μmol of substrate/h at 37 °C.

**Protein Determinations**—Protein concentrations were measured using biuret method (31) and bovine serum albumin as a standard with a BCA protein assay kit (Fierce Chemical Co.).

**Production of Monoclonal Antibodies Against Caprine β-Mannosidase**—The pool of eluted glycoproteins was precipitated with ammonium sulfate as described for the extraction procedure, and the pellets were dissolved in 10 ml of supplemented TBS. These pools of dissolved protein (normal and affected) were repeatedly passed through 43F10S Sepharose columns (20 ml) and checked so that all the 8-mannosidase enzyme was eluted with 4 ml of McIvaine's (35) citric acid-phosphate buffer (pH 5.0) immediately following elution from the column and assayed for enzyme activity. Using this elution procedure 23% of the β-mannosidase activity in the ConA pool (from normal tissues) was recovered in the immunoaffinity pool. The eluted material was stored at ~80 °C prior to SDS-PAGE analysis.

**HPLC**—A Waters 625 LC system equipped with a Lambda-Max 481 spectrophotometer was used with the following columns: a Pharmacia LKB Biotechnology Inc. Mono S HR 5/5 (cation-exchange) column and a Pharmacia LKB Biotechnology Inc. Superose 12 HR 10/30 (gel filtration) column. To concentrate the protein prior to preparative Mono S chromatography the immunoaffinity pool (45 ml) was dialyzed into 1.25 M ammonium sulfate and bound to a 1.0 M low pressure TSK butyl column. The protein bound was eluted with a 10 mM Tris-Cl buffer (pH 6.8) in a 4 ml volume with essentially complete recovery of β-mannosidase activity. This pool was dialyzed into a 10 mM citrate buffer (pH 5.5), 50 mM NaCl, and 10% glycerol and loaded onto the Mono S column. The enzyme was eluted with a NaCl gradient as described previously (29). For gel filtration, the Superose 12 column was equilibrated with 150 mM ammonium carbonate (pH 8.0) buffer, and 200 μl of the immunoaffinity pool was injected. The fractions (0.5 ml) were freeze-dried and resuspended in 50 μl of SDS-PAGE sample buffer.

**SDS-PAGE Analysis**—Gel electrophoresis, gel staining, transfers, and Western analysis were done essentially as described in Ref. 36. The SDS-PAGE sample buffer contained 2.5% (v/v) 2-mercaptoethanol. The second antibodies used in the Western analysis were alkaline phosphatase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG.

**Peptide Mapping by Limited Proteolysis in SDS-PAGE**—Peptide mapping was carried out essentially as described by Cleveland and colleagues (37), as adapted by Polak and Wilson (38). Modifications of this procedure for Polak and Wilson are described in the legend to Fig. 2.

**Glycosylation Studies**— Immunoaffinity-purified β-mannosidase (20 μl, 0.1 μg/ml) was heated (65 °C, 15 min) in the presence of 0.15% SDS and 0.3 M β-mercaptoethanol. Following denaturation the sample was diluted by the addition of 15 μl of 0.077 M potassium phosphate (pH 7.0), 1.5% N-octyl glucoside, 0.065 M EDTA, N-Glycosidase F (0.3 units) or endoglycosidase H (0.004 units) was added and the sample was incubated at 37 °C for 12 h.

**Production of Polyclonal Antiserum**—Immunopurification of caprine β-mannosidase (300 μg) was subjected to preparative SDS-PAGE. Follows gel electrophoresis the gel was stained with a aqueous solution of Coomassie Blue (0.5 g/liter) and destained in water. Gel slices containing the 80- or 90-kDa peptide were processed by repeatedly working the acrylamide through a syringe until it could be easily passed through a 21-gauge needle. The processed acrylamide was emulsified in incomplete Freund's adjuvant prior to injection (34). The gel slice containing the 90-kDa peptide was used to immunize and boost two New Zealand White rabbits. One of the rabbits produced antiserum that reacts with the 90- and 100-kDa peptides plus the 80-kDa peptide which was very likely a minor contaminant in the gel slice. This antiserum is referred to throughout the text as anti-90/100/100. The gel slice containing the 90-kDa peptide was used to raise and boost a rabbit antiserum that reacts with only the 80-kDa peptide. This antiserum is referred to throughout the text as anti-80.

**RESULTS**

**Production and Characterization of mAbs Against Caprine β-Mannosidase**—The fusion and screening of the splea from an immunized mouse generated eight hybridoma clones that produced antibodies capable of precipitating β-mannosidase activity. When used to probe a Western blot of partially purified β-mannosidase (2,000-fold) three mAbs failed to produce detectable signals with the denatured protein but three others detected a 90- and a 100-kDa peptide. mAb 4419 gave the strongest signal in this analysis (data not shown). Evalu-
ation of the specificity of mAb 43F10S toward a panel of lysosomal hydrolases revealed that the antibody could precipitate greater than 95% of the \( \beta \)-mannosidase activity in goat plasma and crude kidney extracts without precipitating detectable levels of \( \alpha \)-mannosidase, \( \alpha \)-glucosidase, \( \alpha \)-fucosidase, or \( \beta \)-hexosaminidase.

**Immunoaffinity Purification of Lysosomal \( \beta \)-Mannosidase**

Following ConA-Sepharose chromatography (29), \( \beta \)-mannosidase was purified by immunoaffinity chromatography using the mAb 43F10S. The immunoaffinity pool contains primarily three peptides (Fig. 1, lane a) as assessed by Coomassie Blue-stained SDS-PAGE. Two of these peptides (100 and 90 kDa) react with the mAb 44D9 and the third peptide (a broad 80-kDa band) does not (Fig. 1, lane b). A small amount of mouse IgG eluted from the immunoaffinity column is detected by the goat anti-mouse IgG polyclonal serum (Fig. 1, lane c). To further address the degree of relatedness between the 100-, 90-, and 80-kDa peptides they were subjected to peptide mapping by limited proteolysis in SDS-PAGE (Fig. 2). Limited proteolysis of the 100- and 90-kDa peptides produced very similar peptide patterns. The 80-kDa peptide pattern does not appear to be related to that of either the 100- or 90-kDa peptides.

**HPLC Purification and Characterization**

For additional purification of the enzyme and to determine whether the 80-kDa peptide is associated with, or required for, \( \beta \)-mannosidase activity, the immunoaffinity pool was subjected to HPLC chromatography.

Gel filtration of immunoaffinity purified \( \beta \)-mannosidase produced two protein peaks (data not shown). The major peak contained approximately 90% of the protein and all of the \( \beta \)-mannosidase activity. SDS-PAGE analysis of selected fractions revealed that the predominant peptides in the fractions corresponding to enzymatic activity were the 90- and 100-kDa peptides. A major portion of the 80-kDa peptide eluted in a minor peak, prior to the enzyme activity, apparently as an oligomer or aggregate.

The elution position of the 100- and 90-kDa peptides from the cation exchange column (Fig. 3B) also corresponds to the activity profile (Fig. 3A), whereas the 80-kDa peak eluted earlier. \( \beta \)-Mannosidase activity is apparently associated with both the 90- and 100-kDa peptides which are partially resolved by Mono S chromatography. Western blot analysis of fraction 18 was performed to identify the smaller peptides which were present in this preparation (data not shown). The 35-kDa peptide whose elution position also corresponds with enzyme activity reacts with anti-80/90/100 polyclonal serum. There is no reactivity of any peptides with anti-80 polyclonal serum, whereas anti-mouse IgG reacted with the 58- and 28-kDa peptides. Therefore, the 58- and 28-kDa peptides are mouse IgG which co-elute with the 90-kDa peptide in the Mono S chromatography. The 35-kDa peptide is immunologically related to the 90- and 100-kDa peptides and may be the result of a small amount of protein degradation.

**Carbohydrate Composition of \( \beta \)-Mannosidase**

The level and type of glycosylation of the 100- and 90-kDa peptides were examined using endoglycosidase H and N-glycosidase F (Fig. 4). N-Glycosidase F, which removes all N-linked oligosaccharides from glycoproteins, reduced the molecular size of the 100- and 90-kDa peptides to 91 and 86 kDa, respectively (Fig. 4, lane 3). Glycosylation of the 90-kDa peptide is mainly high mannose, since endoglycosidase H digestion yielded an approximately 86-kDa peptide (Fig. 4, lane 1). The 100-kDa peptide is more heavily glycosylated and contains complex-type oligosaccharides, since endoglycosidase H digestion reduces the size of the 100-kDa peptide to approximately 96 kDa.

**Comparative Analysis of Affinity-purified \( \beta \)-Mannosidase from Normal and Affected Tissues**

To characterize the defect in goats affected with \( \beta \)-mannosidosis, kidneys from affected goats were examined using endoglycosidase H and N-glycosidase F (Fig. 4). N-Glycosidase F, which removes all N-linked oligosaccharides from glycoproteins, reduced the molecular size of the 100- and 90-kDa peptides to 91 and 86 kDa, respectively (Fig. 4, lane 3). Glycosylation of the 90-kDa peptide is mainly high mannose, since endoglycosidase H digestion yielded an approximately 86-kDa peptide (Fig. 4, lane 1). The 100-kDa peptide is more heavily glycosylated and contains complex-type oligosaccharides, since endoglycosidase H digestion reduces the size of the 100-kDa peptide to approximately 96 kDa.
A multistep dase. 825 units/l. during column loading, 1-ml fractions were collected during elution. The purification procedure. Equivalent amounts of age-acrylamide and protein bands were visualized by silver staining. with endoglycosidase H pools of glycoproteins recovered from this step (normal and control animals were subjected to the first two steps of the purification procedure. Equivalent amounts of age-matched normal (55 g) and affected (52 g) kidney tissue were homogenized, bound, and eluted from ConA-Sepharose. The pools of glycoproteins recovered from this step (normal and affected) contained almost identical levels of protein and α-mannosidase activity. Nearly equivalent amounts of the 80-/mOs peptides are present in the ConA-Sepharose pool from affected and normal kidney tissue prior to and following immunoaffinity-depletion (Fig. 5A). The pools of glycoproteins (normal and affected) from the ConA-Sepharose step were subjected to immunoaffinity purification using two identical analytical sized columns (0.5 ml of resin). Following elution, equivalent volumes of the normal and affected eluate were analyzed by SDS-PAGE. Both silver staining and Western analysis of the immunoaffinity pool from affected tissues indicate that the 90- and 100-kDa peptides are absent (Fig. 5, B and C).

DISCUSSION

An immunoaffinity purification procedure has been developed to prepare purified goat kidney lysosomal β-mannosidase in high yield. This protocol, summarized in Table I, produces an enzyme preparation with a specific activity of 1,190 units/mg of protein with a 26% yield and a 120,000-fold purification. When assessed by silver stained SDS-PAGE or by Western analysis, two peptides (100 and 90 kDa) predominate, and these peptides are associated with β-mannosidase activity in subsequent chromatography. Activity is associated with both the 90- and 100-kDa peptides which were partially resolved by Mono S chromatography. The 90- and 100-kDa peptides both react with anti-β-mannosidase monoclonal antibodies and produce very similar peptide patterns when subjected to limited proteolysis. Deglycosylation of the peptides reveals that they differ in peptide size by approximately 5 kDa. A precursor/product or alternative processing relationship seems plausible and would be consistent with our findings.

When extracts of kidney tissue from goats with β-mannosidase deficiency are subjected to ConA-Sepharose and immunoaffinity chromatography, the 90- and 100-kDa peptides are not detected in the immunoaffinity eluate, as judged by silver staining and Western analysis. This implies that these peptides are either absent in affected tissue or they are structurally altered in such a way that either the concanavalin A
or the mAb 43F10S does not bind to them. The absence of both of these peptides in animals affected by this autosomal recessive disease further substantiates the hypothesis that these peptides have a precursor/product or alternative processing relationship. An alternative hypothesis, that these peptides represent two nonallelic isozymes, is unlikely since car
prine β-mannosidosis is inherited in a simple autosomal recessive manner.

The major contaminants following immunoaffinity chromatography are an 80-kDa peptide and the light and heavy chains of mouse IgG. The 80-kDa peptide is almost completely resolved from the activity profile by the process of gel filtration or Mono S chromatography. The 80-kDa peptide is abundant in the ConA-Sepharose pool and only a very small percent of the peptide is retained by the immunoaffinity column. The amount and size of the 80-kDa peptide is unchanged in affected tissues as detected by Western analysis of the ConA-Sepharose pool. Another peptide of 35 kDa is present in variable amounts and probably represents an in vitro or in vivo proteolytic fragment of the larger 90- or 100-kDa peptide, since it is immunologically related to them.

Goat lysosomal β-mannosidase can now be reproducibly purified to near homogeneity. The enzyme preparation from kidney consists of two related peptides of 100 and 90 kDa. The absence or modification of these peptides in tissue from animals with β-mannosidase deficiency further substantiates their identity.

Acknowledgments — We thank Peggy Bull of the Department of Pathology at Michigan State University for the hybridoma production and her assistance with cell culture. We appreciate the helpful comments of Robert Hausinger, John Wilson, and Steven Triezenberg in their review of the manuscript.

REFERENCES


TABLE I

Summary of the purification of β-mannosidase from normal goat kidney

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>mg</td>
<td>units/mg protein</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>High speed supernatant</td>
<td>2,430</td>
<td>243,000</td>
<td>0.010</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>ConA-Sepharose chromatography</td>
<td>1,700</td>
<td>4,080</td>
<td>0.417</td>
<td>70</td>
<td>42</td>
</tr>
<tr>
<td>Immunoaffinity chromatography</td>
<td>825</td>
<td>1.3</td>
<td>635</td>
<td>34</td>
<td>63,000</td>
</tr>
<tr>
<td>HPLC: Mono S chromatography</td>
<td>643</td>
<td>0.54</td>
<td>1,190</td>
<td>26</td>
<td>120,000</td>
</tr>
</tbody>
</table>