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SUBSTRATE SPECIFICITY AND OTHER PROPERTIES OF THE β-D-GALACTOSIDASE FROM *Aspergillus niger*

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ABSTRACT

β-D-Galactosidase from *Aspergillus niger* was purified by conventional techniques, including the repeated use of chromatography on hydroxylapatite. The final preparation represented a 112-fold purification, with a 22% yield. The specific activity of the purified enzyme was 72 μmol of D-galactose released/min/mg of protein, using *p*-nitrophenyl β-D-galactopyranoside as the substrate. The substrate specificity of the enzyme was studied by using saccharides having structural linkages similar to those found in naturally occurring glycoconjugates. At substrate concentrations of 5mM, the β-D-galactosidase efficiently hydrolyzed β-Gal-1→$OC_6H_4NO_2$-*p*, β-Gal-(1→3)-Gal, β-Gal-(1→3)-β-Gal-1→$OC_6H_4NO_2$-*p*, and β-Gal-(1→3)-α-Gal-1→$OC_6H_4NO_2$-*p*, at rates of 63, 53, 65, and 29 μmol/min/mg of protein, respectively. Slower hydrolysis was observed for β-Gal-(1→4)-β-Glc, β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*, and β-Gal-(1→6)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*, with rates of 10, 13 and 9 μmol/min/mg of protein, respectively. Poorly hydrolyzed, at rates 1/300th of that of β-Gal-1→$OC_6H_4NO_2$-*p*, were synthetic substrates having D-galactose attached β-(1→3)- to either GalNAc or GlcNAc. The K_m value for β-D-galactosidase with β-Gal-(1→4)-β-GlNAc-1→$OC_6H_4NO_2$-*p* was ~20 times that with β-Gal-1→$OC_6H_4NO_2$-*p*. The β-D-galactosidase of *A. niger* has a molecular weight of 300,000, as demonstrated by gel-filtration chromatography. Sodium dodecyl sulfate–poly(acrylamide)-gel electrophoresis indicated a single subunit having a molecular weight of 130,000.

INTRODUCTION

Naturally occurring glycoproteins and glycolipids contain D-galactose in a variety of different linkages. Common D-galactose linkages include D-(1→3), D-(1→4), and β-(1→6) to 2-acetamido-2-deoxy-D-glucose, D-galactose, and 2-acetamido-2-deoxy-D-galactose[1,2]. Purified β-D-galactosidase (EC 3.2.1.23) from

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jack bean[3], *Streptococcus (=Diplococcus) pneumoniae*[4,5], *Aspergillus niger*[6-9], *Clostridium perfringens*[10], and *Escherichia coli*[11] have been routinely used for structural analysis of glycoconjugates[12-15]. These exoenzymes, which liberate D-galactose from the nonreducing terminal of carbohydrates, have strict glycon specificity, and are useful for identifying D-galactose as a structural component of carbohydrates. Unfortunately, comprehensive information concerning the aglycon specificity of these enzymes is lacking.

It has been generally presumed that exoglycosidases have broad, aglycon specificity. However, the results of certain studies suggested that these enzymes also have restricted aglycon specificity[16,17]. For example, the β-D-galactosidase of *S. pneumoniae* was found to hydrolyze β-Gal-(1→4)-GlcNAc, but did not hydrolyze D-galactosides in which the D-galactose was linked β-(1→3) or β-(1→6) to 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, or L-arabinose[13]. Arakawa *et al.*[12] found that the β-D-galactosidase of jack bean also has a preference for the D-galactose of compounds containing β-Gal-(1→4)-GlcNAc. However, depending on the conditions of digestion (concentration of substrate and enzyme), jack-bean β-D-galactosidase can also hydrolyze β-Gal-(1→3)-GlcNAc. Finally, Baenziger and Fiete[18] reported that the D-galactose in a β-(1→4)-GlcNAc-linkage in one of the branches of fetuin is resistant to hydrolysis by the β-D-galactosidase of both jack bean and *S. pneumoniae*. They concluded that the core of this branched glycoprotein restricts the release of D-galactose by these two β-D-galactosidases.

Thus, it appears that the β-D-galactosidases have aglycon specificity that may be further restricted by the structural composition and configuration of the complete glycoconjugate. These studies illustrate the need for information concerning the aglycon specificity of the β-D-galactosidases. Information concerning the aglycon specificity of these enzymes has been difficult to obtain, because the appropriate substrate compounds were not available. Thus, a variety of chemically defined, D-galactose-containing saccharides have now been synthesized, and used for studying the substrate specificity of the β-D-galactosidase from *A. niger*.

In preliminary work[19], it was observed that the crude extract of *A. niger* rapidly hydrolyzes β-Gal-(1→3)-β-Gal-1→$OC_6H_4NO_2$-*p*. Other studies have shown that a crude preparation of *A. niger* hydrolyzes β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*, but not β-Gal-(1→3)-β-GalNAc-1→$OC_6H_4NO_2$-*p*. Thus, it was of interest to determine if the hydrolysis of β-Gal-(1→3)-β-Gal-1→$OC_6H_4NO_2$-*p* and β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* was due to the same or to different enzymes. Therefore, during the enzyme purification process[20], an attempt was made to identify separate β-D-galactosidases. Although the existence of more than one β-D-galactosidase was not demonstrated, an enzyme preparation having high specific activity was produced, and the aglycon specificity of the enzyme was studied.

EXPERIMENTAL

Materials and methods. — The following compounds were prepared as described in the references: β-Gal-(1→3)-β-Gal-1→$OC_6H_4NO_2$ -*p*[21], α- and β-Gal-(1→2)-β-Gal-1→$OC_6H_4NO_2$-*p*[22], β-Gal-(1→6)-β-Gal-1→$OC_6H_4NO_2$-*p*[23], β-Gal-(1→3)- and β-Gal-(1→6)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*[24], β-Gal-(1→3)-β- GalNAc-1→$OC_6H_4NO_2$-*p*[25], β-Gal-(1→4)- and β-Gal-(1→3)-β-GlcNAc-(1→6)-α-Man-1→$OC_6H_4NO_2$-*p* and β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*[26], β-Gal-(1→3)-α-Gal-1→$OC_6H_4NO_2$-*p* and β-Gal-(1→3)-α-GalNAc-1→OPh[27], β-Gal-(1→4)-GlcNAc[28], and β-Gal-(1→3)-GalNAc[29]. β-Gal-(1→3)-Gal was a generous gift from Dr. G. O. Aspinall. β-Gal-(1→4)-Glc and β-Gal-(1→3)-Ara were purchased from Pfanstiehl Laboratories, Inc. All other nitrophenyl glycosides, and a commercially available, purified preparation of *Aspergillus niger* β-D-galactosidase, were obtained from Sigma Chemical Co. A crude enzyme extract from *Aspergillus niger* (Rhozyme HP-150) was purchased from Rohm and Hass, Co., Philadelphia, PA. Asialofetuin was prepared according to Spiro[30]. Ultrafiltration membranes (PM 10) were a product of Amicon. Sepharose-6B, Sephadex G-150, and DEAE-Sephadex A-50 were products of Pharmacia. Ion-exchange resins AG-1 X8 and AG-50W X8, and hydroxylapatite, were from Bio-Rad Laboratories.

Enzyme assays. — Routine, spectrophotometric assays of β-D-galactosidase contained 50mM acetate buffer (pH 4.5), 8mM *p*-nitrophenyl β-D-galactopyranoside, and enzyme; in a final volume of 50 μL. The mixture was incubated for 10–30 min at 37°, and terminated by adding 1 mL of 200mM Na_2CO_3. Absorbance was measured at 400 nm. Other glycosidase assays were performed under the same conditions, with a 5mM concentration of the appropriate nitrophenyl glycoside. Protein was estimated by the method of Lowry *et al.*[31]. One unit was defined as the amount of enzyme which released 1 μmol of the nitrophenol/min. Specific activity was expressed as the number of units/mg of protein.

Enzyme purification. — All purification procedures were performed at 4°. Enzyme preparations were concentrated, and equilibrated with the appropriate buffer, by means of an Amicon pressure-cell equipped with a PM 10 membrane. The β-D-galactosidase from *A. niger* was purified by the procedure of Matta and Bahl[20], with two additional, purification steps. These additional procedures involved the use of a second DEAE-Sephadex A-50 column and a second hydroxylapatite column. The purification procedures used are outlined in Table I. The starting material was 50 g of the crude enzyme preparation (Rhozyme HP-150). The powdered enzyme was extracted, fractionated with ammonium sulfate, and chromatographed on a column of Sephadex G-150 as described[20]. The β-D-galactosidase-rich fractions from the Sephadex G-150 column were pooled, concentrated, and equilibrated with 50mM sodium phosphate buffer (pH 6.8).

The enzyme was loaded onto a DEAE-Sephadex A-50 column (2.4 × 40 cm) equilibrated with 50mM sodium phosphate buffer (pH 6.8). Elution was performed with a continuous gradient between 200 mL of 40mM sodium phosphate (pH 7.6),

TABLE I

PURIFICATION OF β-D-GALACTOSIDASE I FROM *Aspergillus niger*

Step	*Total protein (mg)*	*Total activity (units)*[a]	*Specific activity (units/mg of protein)*	*Purification (-fold)*	*Yield (%)*
Crude extract	9062	5821	0.6	1	100
Ammonium sulfate precipitation	4378	5370	1.2	2	92
Sephadex G-150	755	4121	5.5	9	71
DEAE-Sephadex A-50 (I)	493	3905	7.9	13	67
DEAE-Sephadex A-50 (II)	231	2679	11.6	18	46
Hydroxylapatite (I)	27	1965	72.8	113	34
Hydroxylapatite (II)	18	1293	71.9	112	22

[a] A unit is one μmol of *p*-nitrophenol released per min.

and 200 mL of 300mM NaCl contained in the same buffer. Fractions containing the enzyme were pooled, concentrated, equilibrated with 50mM sodium phosphate buffer, pH 6.8, and loaded onto a second DEAE-Sephadex A-50 column under the conditions used for the first column. For this column, elution was performed with a continuous gradient between 1 L of 40mM sodium phosphate buffer, pH 7.6, and 1 L of 300mM NaCl in the same buffer. Enzyme-containing fractions from this column were pooled, concentrated, and equilibrated with 5mM sodium phosphate buffer, pH 5.8.

Hydroxylapatite chromatography was performed with a column bed (3.2 × 6 cm) as described[20]. The enzyme-rich fractions that were eluted with 600mM sodium phosphate buffer (pH 6.8) were pooled, concentrated, and equilibrated with 300mM sodium phosphate buffer (pH 5.8), and loaded onto a second hydroxylapatite column (3.2 × 6 cm) equilibrated with the same buffer. This column was washed with the starting buffer until no material absorbing at λ 280 nm was detected. The β-D-galactosidase was eluted with 600mM sodium phosphate buffer, pH 5.8. Enzyme-containing fractions were pooled, concentrated, and equilibrated with 5mM acetate buffer, pH 4.5, containing 0.02% of NaN_3, and stored at 4°.

Gel-filtration chromatography. — The molecular weight of purified β-D-galactosidase was ascertained at pH 7.0, as described[32]. The column was loaded with 90 μg of the enzyme, and the elution volume of the enzyme was determined by the standard, β-D-galactosidase assay-procedure.

Electrophoresis. — Poly(acrylamide)-gel electrophoresis of the purified enzyme (9 μg) was performed at 4° in 7.5% gels (0.5 × 6.0 cm) at[33] pH 8.9. Electrophoresis in the presence of sodium dodecyl sulfate was conducted on 5% gels (0.5 × 6.0 cm) at[34] pH 7.0. The following standards, obtained from Sigma, were co-electrophoresed with 9 μg of β-D-galactosidase: thyroglobulin (M_r 330,000), ferritin (220,000), albumin (67,000), catalase (60,000) and lactate dehydrogenase

(36,000). Gels were fixed and stained as described[32].

Kinetics, pH, and temperature studies. — K_m and V_{max} were graphically determined by the method of Lineweaver and Burk[35]. Studies involving pH were conducted with the citrate and phosphate buffer system[36] in the pH range of 2.2 to 8.0. The pH optimum was determined by the standard assay-procedure at various pH values. The pH stability of the enzyme was determined by incubating the enzyme, without substrate, in 5mM buffer for 1 h at various pH values. Substrate and 50mM acetate buffer, pH 4.5, were then added, and the mixture was incubated for 15 min at 37°. The temperature optimum was determined by incubating the standard assay mixture for 15 min at various temperatures. Stability of the enzyme at 65° was determined by incubating the standard-assay mixture, without substrate, for various periods of time at 65°. The enzyme sample was placed on ice, *p*-nitrophenyl β-D-galactopyranoside was added, and the mixture was incubated for 15 min at 37°.

Substrate specificity studies. — Various D-galactose-containing compounds were used as a substrate for β-D-galactosidase, and the enzymically released D-galactose was measured by gas–liquid chromatography of the per(trimethylsilyl) ether. Assays were conducted, as described earlier, with 5mM substrate in a final volume of 0.1 mL. After incubation for 20 min at 37°, the mixture was placed on ice, and transferred to an ion-exchange column of 1 g of Bio-Rad AG-1 X8 (H^+) underlying 0.5 g of Bio-Rad AG-50W X8 (formate). In some cases, the enzyme reaction was terminated by placing the sample in a boiling-water bath for 2 min prior to transferring the mixture to the ion-exchange column. Ion-exchange columns were washed with water (10 mL), to elute the liberated D-galactose. The eluate was evaporated to dryness at 35° in a rotary evaporator, and the D-galactose in the residue was converted into its per(trimethylsilyl) ether as described[37], methyl α-D-mannopyranoside being used as the internal standard. A 1-μL sample was injected into a Bendix 2500 gas–liquid chromatograph equipped with a column (1.83 m × 6.35 mm) containing 3% of SE-30 on Chromosorb WHP (Supelco Inc.; 80–100 mesh). The temperature program consisted of 5 min at 150°, followed by a temperature rate-increase of 5°/min.

RESULTS

Purity. — The β-D-galactosidase from *A. niger* was purified by a series of chromatographic procedures, including hydroxylapatite chromatography[20]. As purity was a major concern for these studies, DEAE-Sephadex A-50 and hydroxylapatite chromatography were each used twice (see Table I). The elution profiles from the Sephadex G-200, DEAE-Sephadex A-50, and hydroxylapatite columns were essentially as reported[20]. The elution profile for the second hydroxylapatite column (see Fig. 1) shows that the β-D-galactosidase is eluted in a sharp peak with 600mM phosphate buffer, pH 5.8. Small amounts of other glycosidases (activity not detectable at the same level as β-D-galactosidase activity) were eluted with 300mM phosphate buffer. The final preparation of β-D-galac-

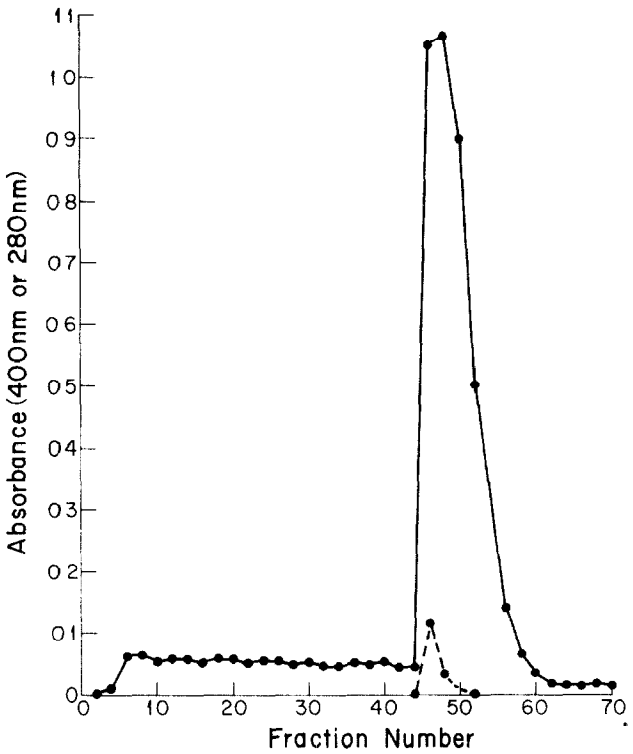

Fig. 1. Elution profile of β-D-galactosidase from the second hydroxylapatite column. [The enzyme was applied, the column was washed with 300mM phosphate buffer, pH 5.8, and 40 fractions (10 mL each) were collected. The buffer concentration was then increased to 600mM. Fractions 45–56 were pooled. Absorbance 280 nm (●---●); enzyme activity (●—●).]

tosidase (fractions 45–56) contained only low levels of other glycosidase activity. Contaminating glycosidases, assayed with the appropriate *p*-nitrophenyl glycosides, and their activity presented as a percentage of the β-D-galactosidase activity, included: α-D-galactosidase, 0.2; β-D-glucosidase, 0.05; α-D-glucosidase, 0.01; β-*N*-acetylglucosaminidase, 0.0; β-D-mannosidase, 0.06; and β-D-xylosidase, 0.0.

The final, purified enzyme-preparation was purified 112-fold over the starting material, and had a specific activity of 72 units/mg of protein (see Table I). The final enzyme-preparation showed, on poly(acrylamide) gels, a single, protein band that coincided with the β-D-galactosidase activity (see Fig. 2). The concentrated, purified enzyme-preparation (64 units/mL) did not show any detectable loss of activity when stored for 6 months at 4°. Enzyme activity was linear with respect to protein, from 0 to 54 μg, and with respect to time, from 0 to 36 min.

Molecular weight. — The molecular weight of the native enzyme as determined by gel filtration was 300,000. The molecular weight determined by sodium dodecyl sulfate–poly(acrylamide) gel-electrophoresis was 135,000 (see Fig. 3).

pH and temperature studies. — The enzyme, assayed with *p*-nitrophenyl β-D-

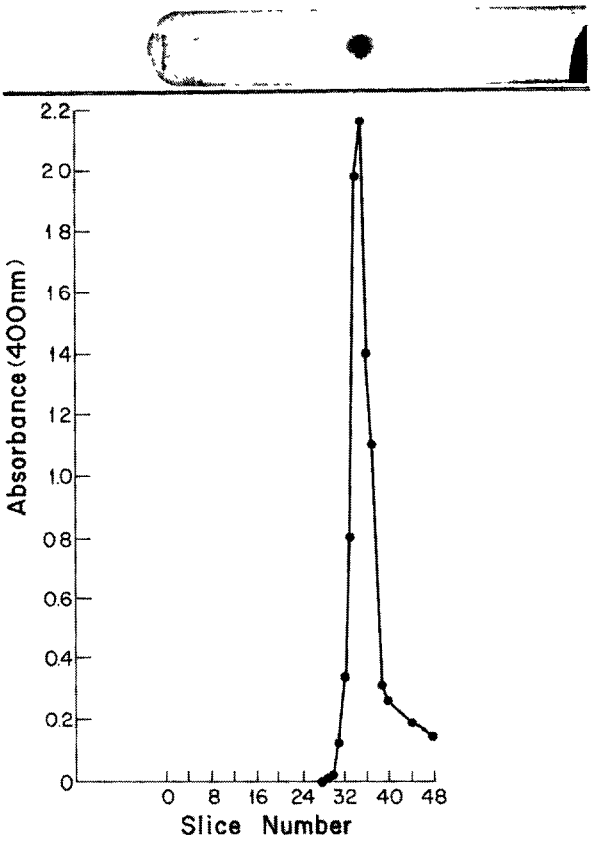

Fig. 2. Poly(acrylamide)-gel electrophoresis of β-D-galactosidase from *A. niger*. [Top panel, electrophoresis of purified β-D-galactosidase (4.5 μg) at pH 8.9. Protein stained with Coomassie Blue. Bottom panel, enzyme activity of a co-electrophoresed gel. This gel was sliced, and each slice was assayed for enzyme by using *p*-nitrophenyl β-D-galactopyranoside.]

galactoside, has optimal activity in the pH range between 4.0 and 4.5. The effect of pH on the stability of the enzyme, incubated for 60 min at 37° without a substrate, indicates that the enzyme is remarkably stable in the pH range of 4 to 7. The optimal temperature for enzyme activity is 65°, and the enzyme has a temperature coefficient (Q_{10}) of 1.9 for the temperature interval of 25 to 35°. Pre-incubation of the enzyme at 65° without substrate caused the enzyme to lose 30% of its activity after 30 min, 44% after 90 min and 60% after 180 min.

Substrate specificity. — The ability of β-D-galactosidase to release D-galactose from compounds containing a D-galactosyl group (at the nonreducing terminal) is shown in Table II. The values for each compound (nmol/min/mg) have been so corrected that the numbers represent only the first D-galactose released from the nonreducing terminal.

Efficiently hydrolyzed were compounds having D-galactose linked β-(1→3)

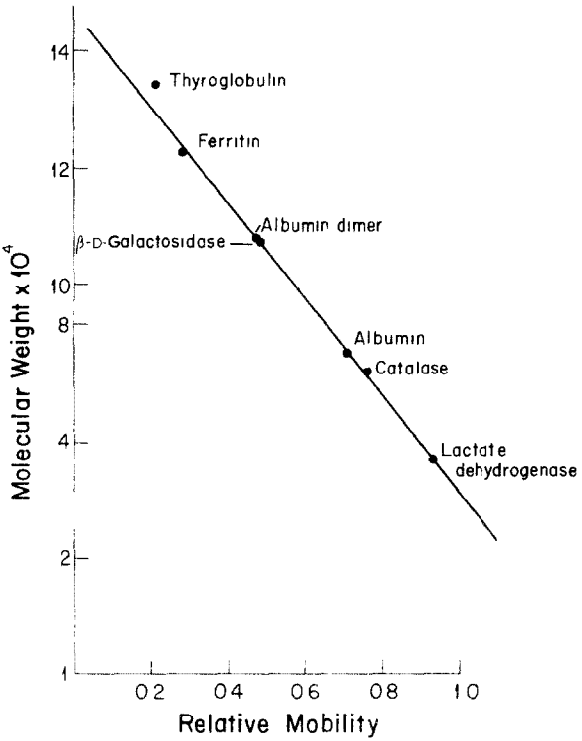

Fig. 3. The relative mobility of β-D-galactosidase (9 μg) and standards, on sodium dodecyl sulfate-poly(acrylamide) gels

to a second D-galactose. Thus, β-Gal-(1→3)-Gal and its β-linked aryl derivative were hydrolyzed at a rate comparable to that of the substrate (β-Gal-1→$OC_6H_4NO_2$-*p*) routinely used to assay β-D-galactosidase. The compound β-Gal-(1→3)-α-Gal linked to an aryl group was hydrolyzed at about half the rate (29.167 μmol/min/mg of protein) for the aforementioned compounds. β-Gal-(1→3)-Ara was hydrolyzed at a rate of 20.833 μmol/min/mg of protein. Compounds with β-D-galactose linked (1→2) or (1→6) to a second D-galactose, such as β-Gal-(1→2)-β-Gal-1→$OC_6H_4NO_2$-*p* and β-Gal-(1→6)-β-Gal-1→$OC_6H_4NO_2$-*p* were hydrolyzed at rates of 9.722 and 3.194 μmol/min/mg of protein, respectively.

Other compounds, having D-galactose linked β-(1→4) or β-(1→6) to D-glucose or 2-acetamido-2-deoxy-D-glucose, were hydrolyzed at variable rates between 8.333 to 14.862 μmol/min/mg of protein (see Table II). Poorly hydrolyzed were compounds with D-galactose linked β-(1→3) to 2-acetamido-2-deoxy-D-glucose or 2-acetamido-2-deoxy-D-galactose. The glycoprotein fetuin with its sialic acid removed was also poorly hydrolyzed, at the rate of 208 nmol/min/mg of protein. Substrate-specificity studies with a commercially available preparation (Sigma) of β-D-galactosidase from *A. niger* showed similar relative hydrolysis rates with these substrates.

TABLE II

SUBSTRATE SPECIFICITY OF β-D-GALACTOSIDASE FROM *Aspergillus niger*

Substrate	Rate of Hydrolysis (nmol/min/mg of protein)[a]
β-Gal-1→$OC_6H_4NO_2$-*o*	85,277
β-Gal-1→$OC_6H_4NO_2$-*p*	62,500
β-Gal-(1→3)-β-Gal-1→$OC_6H_4NO_2$-*p*	65,278
β-Gal-(1→3)-Gal	52,778
β-Gal-(1→3)-α-Gal-1→$OC_6H_4NO_2$-*p*	29,167
β-Gal-(1→2)-β-Gal-1→$OC_6H_4NO_2$-*p*	9,722
β-Gal-(1→6)-β-Gal-1→$OC_6H_4NO_2$-*p*	3,194
β-Gal-(1→3)-β-GalNAc	14
β-Gal-(1→3)-β-GalNAc-1→$OC_6H_4NO_2$-*p*	56
β-Gal-(1→3)-α-GalNAc-1→OC_6H_5	14
β-Gal-(1→3)-α-GalNAc-1→$OC_6H_4NO_2$-*p*	167
β-Gal-(1→3)-β-GalNAc-(1→6)-α-Man-1→$OC_6H_4NO_2$-*p*	131
β-Gal-(1→6)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*	8,986
β-Gal-(1→4)-GlcNAc	8,333
β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*	13,005
β-Gal-(1→4)-β-GlcNAc-(1→6)-α-Man-1→$OC_6H_4NO_2$-*p*	14,862
β-Gal-(1→4)-Glc	9,722
β-Gal-(1→3)-Ara	20,833
α-Gal-1→$OC_6H_4NO_2$-*p*	97
α-Gal-(1→2)-β-Gal-1→$OC_6H_4NO_2$-*p*	56
Asialofetuin	208

[a]This value represents only the D-galactose released from the nonreducing terminal.

The high K_m value (25.1 mM) for this enzyme with β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* indicates that this substrate binds poorly to the enzyme (see Table III). However, at saturating levels, the compound is hydrolyzed efficiently (see Table III). The K_m value for β-Gal-(1→3)-Gal (4.4mM) is comparable to that of β-Gal-1→$OC_6H_4NO_2$-*p*, the substrate routinely used for the assay of β-D-galactosidase. The V_{max} for β-Gal-(1→3)-α-Gal-1→$OC_6H_4NO_2$-*p* is about half that found for β-Gal-1→$OC_6H_4NO_2$-*p*.

TABLE III

KINETIC CONSTANTS FOR *Aspergillus niger* β-D-GALACTOSIDASE WITH VARIOUS SUBSTRATES

Substrate	V_{max} *(μmol/min/mg of protein)*	K_m *(mM)*
β-Gal-1→$OC_6H_4NO_2$-*p*	76.4	1.2
β-Gal-(1→3)-Gal	94.4	4.3
β-Gal-(1→3)-α-Gal-1→$OC_6H_4NO_2$-*p*	56.8	5.5
β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p*	80.7	25.1

DISCUSSION

The β-D-galactosidase of *A. niger*, described herein, was purified 112-fold over the starting material, with a 22% yield. It appears that comparable purification could have been achieved had the first DEAE-Sephadex A-50 and the second hydroxylapatite chromatography steps been omitted. However, extensive washing of the hydroxylapatite column with 300mM buffer prior to elution of the enzyme is important for removal of traces of contaminating glycosidases.

The specific activity of the final preparation was 72 μmol of D-galactose/min/mg of protein, using *p*-nitrophenyl β-D-galactopyranoside as the substrate. The specific activity of this preparation is higher than that reported for the enzyme when similar purification procedures, but without hydroxylapatite chromatography, were used[6-8]. The specific activity of a commercial preparation (Sigma) of β-D-galactosidase from *A. niger* was 12 μmol/min/mg of protein. The enzyme properties (pH optimum, pH stability, and kinetic constants) of the preparation described herein were comparable to those reported[6,7], indicating that the enzyme was not altered by hydroxylapatite chromatography.

An interesting result from these studies is the preference of the *A. niger* enzyme for the β-Gal-(1→3)-Gal compound. This compound is hydrolyzed more efficiently than the structurally similar compound having (1→2) or (1→6) linkage to D-galactose, indicating high specificity for the β-Gal-(1→3)-Gal linkage. Furthermore, as β-Gal-(1→3)-GlcNAc and β-Gal-(1→3)-GalNAc were poorly hydrolyzed, the *A. niger* enzyme has a preference for D-galactose as the β-(1→3)-linked, penultimate sugar. The β-Gal-(1→3)-Gal structural linkage is found in glycolipids of human erythrocytes[38], in dermatan sulfate[39], and in snail galactans[40]. Thus, β-D-galactosidase from *A. niger* should efficiently hydrolyze these naturally occurring linkage-structures.

Because there is a preponderance of D-galactose that is linked β-(1→4) and β-(1→3) to 2-acetamido-2-deoxy-D-glucose in glycoconjugates, the activity of β-D-galactosidases toward these linkages is particularly interesting. The β-D-galactosidase of *A. niger* has a preference for β-Gal-(1→4)-GlcNAc compared to the β-(1→3)-linked disaccharide. However, the enzyme hydrolyzes the β-(1→6)-linked disaccharides at a rate almost the same as that at which β-(1→4)-linked are cleaved. In contrast to these results with β-D-galactosidase from *A. niger*, Paulson *et al.*[13] reported that the β-D-galactosidase from *S. pneumoniae* hydrolyzes β-Gal-(1→4)-GlcNAc, but does not hydrolyze the β-(1→6)-linked disaccharide. Furthermore, β-Gal-(1→3)-Ara was not hydrolyzed by the enzyme of *S. pneumoniae*, but was hydrolyzed by the enzyme from *A. niger*. Thus, the aglycon specificity of *A. niger* β-D-galactosidase is not as strict as that of the enzyme of *S. pneumoniae*. The inability of the *A. niger* enzyme to cleave substrates containing β-Gal-(1→3)-GlcNAc and β-Gal-(1→3)-GalNAc is comparable to that reported for the β-D-galactosidase of *S. pneumoniae*[13] and jack bean[12]. The inability of these β-D-galactosidases to cleave these compounds indicates that the *N*-acetyl group on the sec-

ond carbon atom hinders the hydrolysis of these compounds. In contrast to these results, the β-D-galactosidases of bovine testis efficiently hydrolyzes[41] β-Gal-(1→3)-GlcNAc and β-Gal-(1→3)-GalNAc.

Kinetic studies on the action of *A. niger* β-D-galactosidase with β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* indicated that the enzyme has a low affinity for this substrate, compared to β-Gal-1→$OC_6H_4NO_2$-*p*, but hydrolysis of this substrate at saturation (V_{max}) is very efficient. High K_m values of 20 and 50mM with lactose were reported for the β-D-galactosidases of *A. niger*[8] and human liver[42], respectively. Thus, in comparison to its activity towards *p*-nitrophenyl β-D-galactoside and β-Gal-(1→3)-Gal, the linkage naturally occurring in β-Gal-(1→4)-β-GlcNAc is hydrolyzed at a lower rate when the substrate concentration is low, but the β-Gal-(1→4)-GlcNAc linkage will be effectively hydrolyzed if the concentration of the substrate or enzyme is high. Indeed, with naturally occurring glycoproteins, for structural studies which employed *A. niger* or *Phaseolus vulgaris* β-D-galactosidase, a high concentration of enzyme (units) was used[15]. The amount of enzyme used was ~100 times that necessary for rapid hydrolysis of *p*-nitrophenyl β-D-galactoside. Thus, although fetuin was poorly hydrolyzed compared to Gal-β-(1→3)-Gal, almost complete hydrolysis should be possible if a higher concentration of the enzyme is used.

In general, aryl disaccharides were hydrolyzed 20–30% faster than their respective, simple disaccharides. Thus, the aryl compound β-Gal-(1→4)-β-GlcNAc-1→$OC_6H_4NO_2$-*p* was hydrolyzed at a rate of 13 μmol/min/mg of protein compared to 8 μmol/min/mg of protein for the simple disaccharide. Furthermore, β-Gal-(1→4)-β-GlcNAc-(1→6)-α-Man-1→$OC_6H_4NO_2$-*p* is hydrolyzed faster than the compound lacking D-mannose. These results indicate that the rate of hydrolysis of compounds having the linkage β-Gal-(1→4)-GlcNAc is dependent on the type and number of residues attached to the anomeric carbon atom of GlcNAc.

The β-D-galactosidase from *A. niger* would not be useful for hydrolyzing oligosaccharides containing D-galactose that is linked β-(1→3) to either 2-acetamido-2-deoxy-D-glucose or 2-acetamido-2-deoxy-D-galactose. Indeed, this enzyme might be useful for detecting the structural linkage of saccharides, as the β-Gal-(1→3) linkage to 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-galactose is hydrolyzed at 1/1000th and 1/100th the rate for β-Gal-(1→3)-Gal and β-Gal-(1→4)-GlcNAc, respectively. However, as others have advised, caution must be exercised in the interpretation of structural study data based only on relative rates of cleavage.

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