0273-2289/93/3940—0213$03.60

Isolation and Characterization of Two Forms of β-D-Glucosidase from *Aspergillus niger*

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ABSTRACT

β-D-glucosidase purified from commercial preparations of clarified culture broth of *Aspergillus niger* (Novo SP188) was shown to elute as two distinct species during analytical anion-exchange chromatography (AEC). However, the two enzyme forms behaved identically on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), high-performance size-exclusion chromatography (HPSEC), and isoelectric focusing. Also, the N-terminal amino acid sequence, amino acid composition, fingerprint of tryptic-digest peptides, circular dichroism spectra, and reaction kinetics appear identical for these forms. This feature of the *A. niger* enzyme is distinctly different from β-D-glucosidase isozymes reported from other sources, where multiple forms tend to differ in molecular weight and/or isoelectric pH. Michaelis-Menten kinetic analysis also gave comparable results for the two forms. The distinct behavior on AEC was explained by considering the differences in N-linked carbohydrates liberated from both species following treatment with endoglycosidase H or F.

Index Entries: β-D-glucosidases; *A. niger;* glycoproteins; endoglycosidase H.

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INTRODUCTION

The cost of cellulase enzymes and the efficiency of the saccharification process are of great interest not only for separate hydrolysis and fermentation (SHF) applications (*1*), but also for simultaneous saccharification and fermentation (SSF) processes (*2,3*). In SSFs, ethanol is produced by concurrent saccharification and yeast fermentation, utilizing the glucose produced from the hydrolysis of the cellulosic content in biomass. The efficient hydrolysis of cellobiose to glucose is, therefore, essential for the successful application of SSF and SHF technologies.

Cellulase enzymes act synergistically on cellulose to produce cellobiose and glucose by cleaving the β-1,4 glucosidic linkages in the polymer chain. With fungal and many bacterial cellulases, this saccharification is enhanced by supplementation with β-D-glucosidase (EC 3.2.1.21), which hydrolyzes cellobiose selectively to glucose (*4,5*). β-D-glucosidase thus provides a key catalytic activity to cellulase preparations. Apart from providing the glucose feedstock for downstream processing (i.e., fermentations), this enzyme also reduces the powerful end product inhibition caused by cellobiose buildup in fungal cellulase digestion systems (*6,7*). Because most *Trichoderma reesei* cellulase systems secrete little β-D-glucosidase, augmentation with the enzyme from *A. niger* is often necessary to obtain the desired performance in saccharification.

The β-D-glucosidase from *A. niger* has been often used for such processes; however, this enzyme has not been the subject of a full biochemical characterization. Scientists in the area (*8–10*) have long been aware that the purification of β-D-glucosidase from *A. niger* by anion-exchange chromatography leads to the discovery of two (or more) apparent forms of the enzyme. The authors' work with the immobilization of this enzyme on anion-exchange resins further pointed to the need to understand better these different forms. For these reasons, the task of characterizing both forms of *A. niger* β-D-glucosidase by a wide array of biochemical methods was undertaken. In the present study, two forms of β-D-glucosidase, referred to here as βGI and βGII, were purified from NOVO SP188 and subjected to a broad spectrum of biophysical and kinetic analyses.

EXPERIMENTAL

Substrates, Enzymes, Buffers, and Reagents

The *p*-nitrophenyl-β-D-glucoside (pNPG) used in this study was obtained from Sigma Chemical Company (St. Lousi, MO). β-D-glucosidase was purified from commercial preparations of *A. niger* culture broth. Novozym SP188 (Novo Ltd., Danvers, OH, batch DCN-01). Endoglycosidase H (EC 3.2.1.96) and endoglycosidase F were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). All buffer components

and salts used were reagent grade, and obtained from either Sigma Chemical Company or Fisher Scientific (Pittsburgh, PA). Unless stated otherwise, all experiments were performed in 10 m*M* sodium acetate buffer, pH 5.0.

Enzyme Purification

The purified enzyme used in this study was prepared by a sequential high-performance size-exclusion (HPSEC) and ion-exchange chromatography (HPIEC) procedure previously reported by Baker et al. (*11*). This approach yields a β-D-glucosidase preparation of approx 99% purity by SDS-PAGE.

A Pharmacia BioPilot Column Chromatography system was used to perform large-scale size-exclusion chromatography (SEC) with an 11.3×90 cm BioProcess column packed with Sephacryl S-200 HR gel. HPSEC and HPIEC were conducted with Pharmacia Superose 6 and 12 (HR 10/30) and Mono-Q (HR 5/5) columns, respectively, equipped with Beckman model 520 system controller and Beckman model 110B high-performance liquid chromatography (HPLC) pumps. β-D-glucosidase preparations were concentrated and dialyzed with an Amicon model DC-2 Hollow Fiber Ultraconcentrator equipped with H1P10-20 or H1P100-20 cartridges.

Enzyme Assays

β-D-glucosidase activity was determined according to the general method of Wood (*12*) as aryl-β-glucosidase activity by the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside. The concentration of *p*-nitrophenol (pNPOH) was determined from the extinction at 410 nm under alkaline conditions induced by the addition of 2*M* Na_2CO_3 at the end of the assay. One unit of activity was defined as that amount of enzyme that catalyzes the cleavage of 1.0 μmol substrate/min at 50°C. Apparent K_m and V_{max} values were determined at 50°C using pNPG as substrate in the concentration range of 0.1–10 m*M*. The protein concentration in the reaction mixture was approx 1 μg/mL. Michaelis-Menten kinetics were analyzed with the ENZFIT software package from Elsevier Publishers. Protein determinations followed the modified Lowry total protein assay of Polachek and Cabib (*13*).

SDS-PAGE, Isoelectric Focusing (IEF), and Electrophoretic Titration Curve Analysis

These electrophoretic analyses of mixed and purified βGI and βGII were performed with the Pharmacia Phast System following procedures described in the Phast System Users Manual, Separation Technique File No. 100 (56-1605-00). SDS-PAGE used Phastgel 8–25% acrylamide gradient gels, and analytical IEF used PhastGel IEF gels, pH 4.0–6.5. Electrophoretic titration curve analysis was done using Pharmacia PhastGel IEF 3-9 gels.

Analytical Ultracentrifugation

Sedimentation equilibrium studies were carried out on samples of enzyme that had been dialyzed against acetate buffer with 100 m*M* NaCl. After dialysis, the enzyme concentration was determined by the Lowry assay to be approx 1 mg/mL. Meniscus depletion sedimentation equilibrium experiments were performed on a Beckman model E ultracentrifuge equipped with an electronic speed control and interference optics. The 3-mm fluid-column, high-speed method of Yphantis (*14*) and Wales (*15*) was performed at 20°C with aluminum-filled epon double-sector cells and an AN-D rotor. Using this method, the temperature was allowed to "coast" during the time required to reach equilibrium. The partial specific volume of 0.733 mL/g was calculated for *A. niger* β-D-glucosidase by summing the products of the weight fractions of each amino acid and its corresponding specific volume following the method of Cohn and Edsall (*16*). The partial specific volume was not corrected for carbohydrate content. Photographs taken of the Rayleigh interference optical system were recorded on Kodak Spectroscopic type II-G plates and analyzed with a Nikon model V12 microcomparator with MC-102 electronic digitizer. Equilibrium fringe patterns were measured at both 10,000 and 11,000 rpm.

Circular Dichroism (CD)

CD spectra were obtained on a JASCO (Easton, MD) model J-600 spectropolarimeter with jacketed cells thermostatted to 25°C. A 0.05-cm pathlength cell and a protein concentration of approx 0.25 mg/mL were used to collect peptide CD data. Primary experimental data were processed by a data-smoothing algorithm (JASCO). Chou-Fasman and other protein secondary-structure prediction calculations were performed with PC software from DNAStar, Inc. (Madison, WI).

Differential Scanning Microcalorimetry (DSC)

Denaturation thermograms were obtained using a Microcal MC-2 Scanning Calorimeter (Microcal, Northampton, MA), interfaced through a DT 2801 A/D converter to an IBM PC-XT microcomputer. Instrument control and data acquisition were by means of the DA-2 software package (Microcal). The sample cell capacity is 1.13 mL, and runs were made with an overpressure of 30 psig (N_2), at scan rates of either 0.5 or 1.0 deg/min. Protein samples were concentrated and exchanged into the buffer to be used in each DSC run by extensive diafiltration against the buffer using Amicon-stirred cells and PM-10 ultraconcentration membranes with 45 psig nitrogen pressure. The protein sample was then degassed under aspirator vacuum for a minimum of 20 min, with stirring, in order to remove excess dissolved gas forced into the solution during the diafiltration step. A sample of diafiltration buffer, to be used in filling the reference cell of the calorimeter, was given identical treatment. Buffers used were 50 m*M* acetate, pH 5.0.

N-Terminal Sequence and Amino Acid Analysis

N-terminal sequence analysis of βGI and βGII was performed by Edman degradation using an Applied Biosystems (Foster City, CA) Gas Phase Sequencer model 470 and an on-line phenylisothiocyanate (PTH) amino acid analyzer following methods described by Applied Biosystems. Samples for amino acid analysis were dialyzed against distilled water and hydrolyzed with constant boiling HCl in evacuated and sealed tubes at 110°C for 24, 48, and 72 h. After hydrolysis, the amino acids were derivatized with (dimethylamino) azobenzenesulfonyl chloride (DABS) following the procedure of Knecht and Chang (*17*) and analyzed by reverse-phase HPLC. Cysteine was determined as cysteic acid after performic acid oxidation of the enzyme.

Trypsin Digestion and Peptide Mapping

Trypsin digestions were performed in a molar ratio of trypsin to substrate (i.e., βGI or βGII) of 1:30 in a vol of 1 mL. A control of trypsin without substrate was also examined to identify peptides from autolysis. Digestions with trypsin were run at pH 8 and 37°C following the procedures of Pastuszyn et al. (*18*). Tryptic peptides were separated on a Vydac C_8 wide-pore, reverse-phase 10×4.6 cm column with detection at 214 nm. After injection of a 40-μg sample of hydrolyzate, a gradient of 0–60% B was run in 60 min; buffer A was 0.1% TFA (trifluoroacetic acid), and buffer B was acetonitrile.

Carbohydrate Composition Analysis

Neutral and amino sugars from HCl hydrolyzates of βGI and βGII were analyzed by HPLC using a Dionex (Sunnyvale, CA) Ion PAC column and pulsed amperometric detector at the Carbohydrate Analysis Facility, University of Virginia, Charlottesville, VA.

Treatment with Endoglycosidase H and F

Two-hundred-and-fifty-microgram samples of β-D-glucosidase preparations containing both forms were incubated at 37°C for 10–12 h with 0.05 U of endoglycosidase H (or F) in a total vol of 200–300 μL. Incubation buffer was 50 m*M* acetate, pH 5.5. This digestion was then injected directly onto an equilibrated Mono-Q column and chromatographed following the standard HPIEC protocol used in purification of individual forms.

RESULTS AND DISCUSSION

Evidence for Conserved Peptides

Figure 1 clearly shows the HPIEC elution profile typically found for β-D-glucosidase purified from *A. niger* growth supernatants; i.e., two

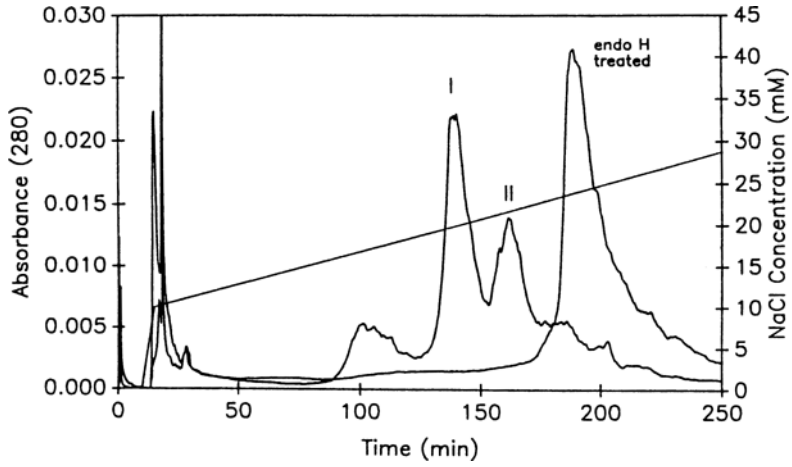

Fig. 1. Typical high-performance anion-exchange chromatogram showing elution of β-D-glucosidase from *A. niger* in two forms. Form I elutes at 260 m*M* NaCl and form II elutes at 290 m*M* NaCl in 10 m*M* acetate buffer. The second elution profile shown resulted from the injection of a sample of mixed forms after treatment with endoglycosidase H.

major peaks with near baseline separation, both yielding highly active enzyme. Although the HPIEC presented in this study was based on Pharmacia Mono-Q, a crosslinked dextran, a similar profile was observed in the authors' laboratory using a noncarbohydrate-based ion-exchange packing material, Synchropak AX-300 (not shown). Thus, interaction of the column support material with eluting enzyme is not a likely contribution to this unique elution behavior.

What the authors believe to be convincing evidence that these two forms of the *A. niger* enzyme contain the same polypeptide component has been compiled. Primary structure was investigated first. Both the N-terminal peptide sequence (SELAYSPPYYPSPWANGQTD-AENY—) and the amino acid composition were also found to be identical (within experimental error) for both forms (Table 1). A tryptic digestion analyzed by wide-pore, reverse-phase chromatography also showed essentially identical patterns of peptide fragments (i.e., match of 36 identifiable eluting peptide fragments from βGI with 40 from βGII) (not shown). Although somewhat less useful for showing fine differences, protein molecular weight was also carefully examined. SDS-gel electrophoresis analysis (Fig. 2) yielded one band for each purified enzyme form, each demonstrating an identical mobility on the gel. When a mixture of the two forms of the enzyme was examined (i.e., a crude SEC fraction), only one band was observed at the correct gel position (e.g., not two closely migrating bands). Also, molecular-weight values determined from these gels agree well with those obtained from sedimentation equilibrium experiments (Table 2). The average of the values from the present study

Table 1
Amino Acid Composition of Two Forms of *A. niger* β-D-Glucosidase

Residue	Residues/1000	
	Form I	Form II
D	173	172.5
Q	111	106
C	0	0
S	80.5	82
G	99	98.5
H	12	11.5
T	63	64
A	97	98.5
R	41.5	41.5
P	59	59.5
Y	18	15
V	57	58
M	12	12
I	32	33.5
L	68	69
F	26	26
K	29	29.5

(119,600 dalton) is surprisingly close to the value reported from gel filtration (120,000 dalton) by Shoseyov et al. (*19*) for the same enzyme system (i.e., *A. niger* β-D-glucosidase). Many other β-D-glucosidase are considerably higher in mol wt and range from 41,000 to 350,000 (*20*).

An indirect method of analysis of secondary structure, CD, was used to analyze further these two chromatographic forms. Although arguably insensitive to fine differences in structure, both forms appear identical in the content of specific secondary features using Chou-Fasman estimation (Table 2). The *A. niger* enzyme shows predicable structure in the far UV or peptide CD region, with negative maxima at 210 and 220 nm (Fig. 3).

In Fig. 4, the thermal denaturation of the two purified enzyme forms is analyzed by differential scanning microcalorimetry. Both forms show significant scan rate independence of the denaturation temperature, which could be consistent with an extremely rapid, reversible protein unfolding occurring around 72°C. Again, no gross differences in structure are indicated. Kinetic values found for the hydrolysis of pNPG by both purified forms (shown in Table 2) indicate significant similarity. Also, these purified enzymes show a slight increase in substrate specificity (i.e., lower values for K_m) than found by Dekker for the crude preparation (*21*).

Because the two forms of the enzyme were separated on ion-exchange chromatography, it is possible that point mutation may provide one form

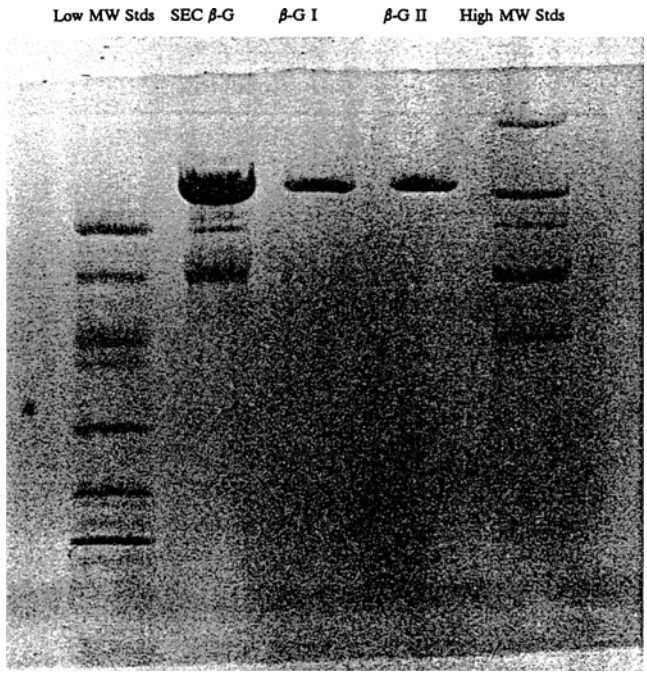

Fig. 2. SDS-PAGE of the SEC-purified enzyme (overloaded in lane 2), ion-exchange form I (lane 3), and ion-exchange form II (lane 4). Lanes 1 and 5 were loaded with low- and high-molecular-weight standards, respectively, from Sigma Chemical. Electrophoresis was performed with a Pharmacia Phast Tris/SDS gel system.

with a slightly different net charge at the pH of elution and thus result in fractionation. However, the IEF experiments shown in Fig. 5A clearly demonstrate that the mixture of forms focuses as a single band. The pH titration curve shown in Fig. 5B also demonstrates no splitting of components at pH extremes.

Evidence for Glycosylation-Induced Elution Effects from AEC

These data lead to the conclusion that it is highly unlikely that these enzyme forms stem from different gene products. The only basis for distinction between the two is the different elution from anion-exchange resin. Furthermore, the additional possibility of artifactual elution from one type of resin is, in this case, remote, because separation of these forms has been observed in the authors' laboratories from both a silica-based resin (Synchropak AX-300) and a dextran-based resin (Pharmacia Mono Q). Further light is shed on the problem by the result of endoglycosidase treatments. The fraction containing a mixture of both forms of the

Table 2
Kinetic and Physicochemical Characteristics
of Purified and Mixed β-D-Glucosidase from *A. niger*

	Form I[a]	Form II	Mixed fraction[b]
Molecular weight by sedimentation equilibrium[c]	ND	ND	117,400 ± 3600
Molecular weight by SDS-PAGE[d]	ND	ND	121,900 ± 4800 (one band)
Isoelectric pH	4.2	4.2	4.2 (one band)
K_m (pNPG)	0.47 m*M*	0.36 m*M*	0.30 m*M*
V_{max} (μmol $min^{-1}mg^{-1}$)	217	211	185
Specific activity (pNPG U mg^{-1})	90	95	84
Secondary structure estimated from CD:			
% α-Helix	0	0	ND[e]
% β-Sheet	80	80	ND[e]
% β-Turn	5	5	ND[e]
% Random Coil	15	15	ND[e]

[a]Purified forms from AEC using Pharmacia Mono-Q.
[b]Fraction taken from SEC exhibiting pNPG hydrolyzing activity.
[c]From meniscus depletion sedimentation equilibrium: 113,800 @ 10K rpm and 121,000 @ 11K rpm.
[d]Average from five runs; all showed one band for mixed forms.
[e]ND, not determined.

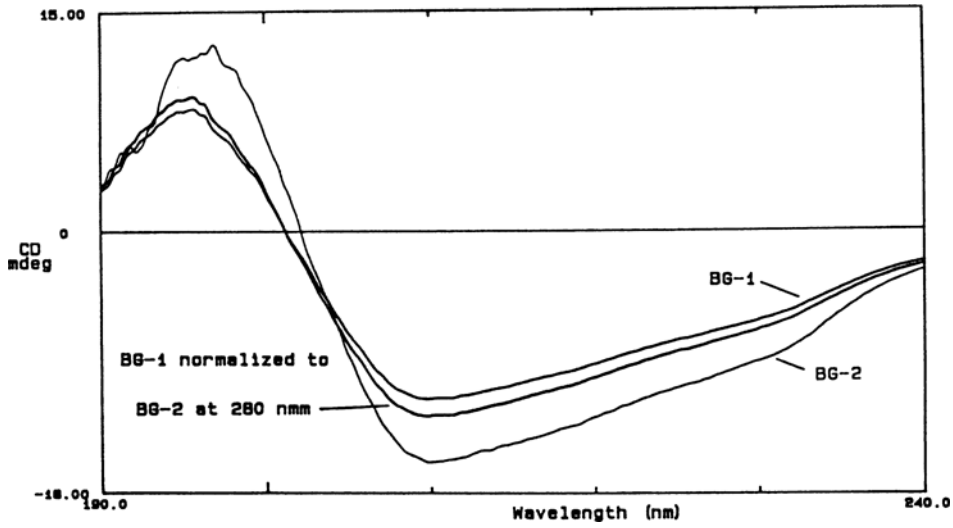

Fig. 3. CD spectra of the β-D-glucosidase forms I and II.

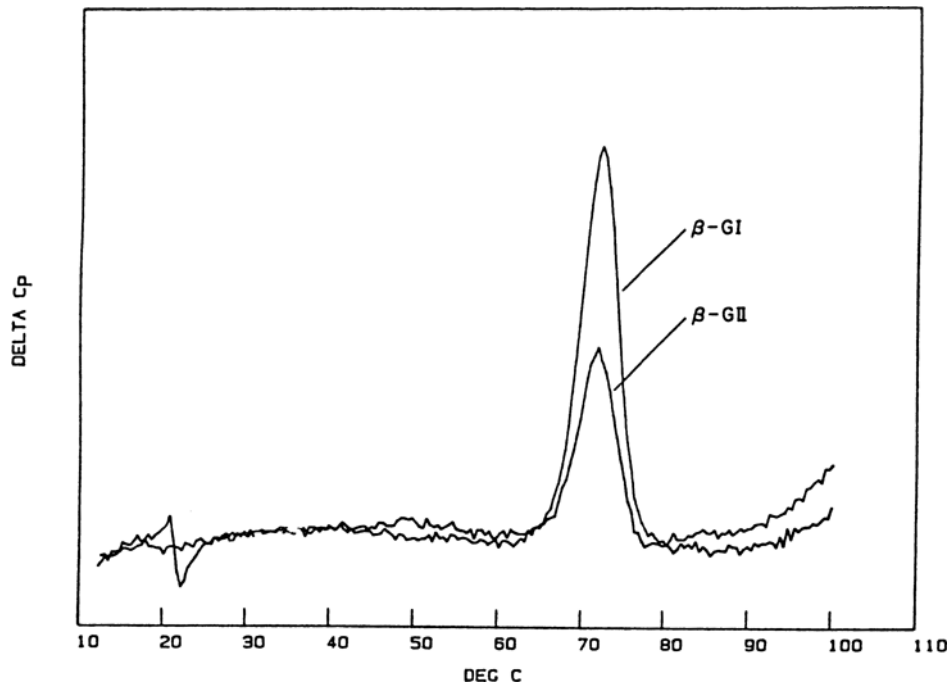

Fig. 4. DSC of the two forms of β-D-glucosidase in 10 m*M* acetate buffer, pH 5.5. Data were collected with a Microcal MC-2 equipped with computer control.

enzyme was incubated with endoglycosidase H and F in separate experiments. Elution of the enzyme digest from a Mono-Q column (shown in Fig. 1 for endoglycosidase H treatment) clearly shows the collapse of the two chromatographic forms to a single species. Similar results were found for endoglycosidase F treatments.

The effects of enzymatic deglycosylation on the AEC profile provide strong evidence that the separation of the two forms by AEC is rooted in the differences between their respective glycosylation either in the (measured) different total quantities of carbohydrate, (possible) differences in the points of attachment to the protein, or both. Given the fact that no sialic acids were found in either chromatographic form (Table 3) and assuming (as is quite likely) that the glucosamine observed (Table 3) is derived during HCl hydrolysis from sugar residues present as *N*-acetylated glucosamine moieties in the native proteins, the glycosylation of both chromatographic forms may well consist of neutral carbohydrate. If this is true, the chromatographic separation of the two forms is probably explained by the fact that in the two forms, the close approach of the charged surface groups of the peptide portion of the molecule to oppositely charged groups on the packing material is hindered to different extents by the presence of neutral glycosylation.

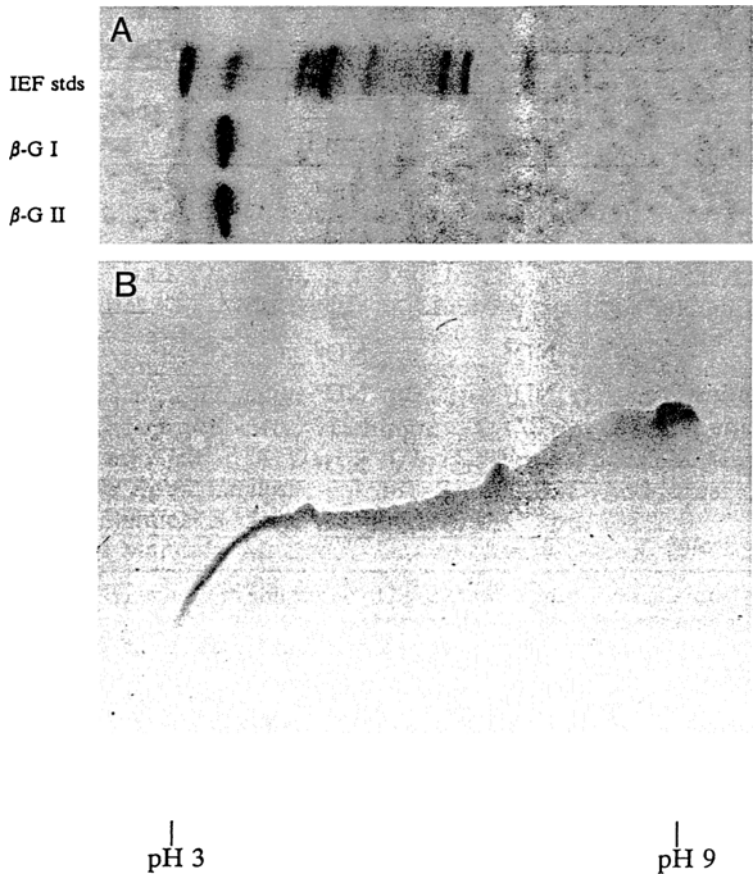

Fig. 5. Plate A shows the isoelectric focusing of forms I and II following purification by AEC. The right lane of the gel (shown at the top of the figure) was loaded with broad-range p*I* standards. Plate B shows the isoelectric titration of an equimolar mixture of forms I and II. A Pharmacia IEF gel, pH 4–6.5, was used with the Phast System.

Protein molecules cannot always be adequately represented as point charges equal to their net charges under a given set of conditions; the charged groups on the exterior of proteins have definite distributions about quite appreciable diameters, and these distributions are by no means always symmetrical (*22*). To a far greater extent than the other methods of analysis used here, ion-exchange chromatography is sensitive to macromolecular-level charge distributions, specifically expressed as attractions between charged packing materials and local patches of opposite charge on the protein, as well as by the net charge on the protein as a whole (*23,24*). One may envision steric hindrance of the close approach of a single charged patch on the protein to the packing material, as by a

Table 3
Oligosaccharide Analyis of *A. Niger* β-D-Glucosidase

Neutral sugars	Mol sugar/mol protein[a]		Oligosaccharide I and II mol/sample[b]
	Form I	Form II	
Inositol	ND[d]	ND[d]	ND[d]
Fucose	ND[d]	ND[d]	ND[d]
Galactose	15	4.4	117
Glucose	4.5	2.5	236
Mannose	70	24	1450
Xylose	ND[d]	ND[d]	ND[d]
Galactosamine	ND[d]	ND[d]	ND[d]
Glucosamine	27	21	235
Sialic acids	ND[d]	ND[d]	ND[d]
Total	117	52	
%Carbohydrate[c]	17%	7.7%	

[a] Aliquots from the same sample were used to determine mol sugar (HCl digest) and mol protein (from amino acid composition analysis).
[b] Polysaccharide fraction obtained from ultrafiltration filtrate (Amicon PM-10) of an endoglycosidase-H-treated aliquot containing both enzyme forms.
[c] Calculated using 121,000-dalton mol wt.
[d] ND, not detected.

sizable neutral carbohydrate chain attached nearby in one chromatographic form and absent in the other (i.e., 90 vs 31 mol sugar/mol protein for form I and form II, respectively). Such steric shielding by carbohydrate in one form would result in that form being eluted earlier than the other. Removal of the carbohydrate from both would eliminate the chromatographic difference between the two forms. This is exactly what is seen in Fig. 1. In fact, inasmuch as the single peak found for the deglycosylated enzyme mixture elutes considerably later than the peak for either native enzyme form, it would appear that both native (glycosylated) forms are shielded significantly from interaction with the packing material; the difference in chromatographic behavior is attributable to a difference in the degree of shielding.

ACKNOWLEDGMENTS

This work was funded by the Ethanol from Biomass Program of the Biofuels Systems Division of the US Department of Energy. The authors also wish to thank John Shannon, Susan Hefler, Linda Beggerly, and Kuni Tatsumoto for valuable technical assistance.

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