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PURIFICATION OF ENDO-*N*-ACETYL-β-D-GLUCOSAMINIDASE H BY SUBSTRATE-AFFINITY CHROMATOGRAPHY*

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ABSTRACT

Endo-*N*-acetyl-β-D-glucosaminidase H (Endo H) was purified to homogeneity (3000-fold) from a culture filtrate of *Streptomyces plicatus*. The key step was substrate-affinity chromatography, which afforded a 1000-fold purification and yielded a protease- and exoglycosidase-free preparation of Endo H. Proteins from the crude sample were applied to the substrate-affinity column, consisting of yeast-invertase glycopeptides bound to Sepharose-immobilized concanavalin A. After washing off the unbound proteins, Endo H was quantitatively eluted by methyl α-D-mannopyranoside. Various conditions were tested to achieve an optimal binding of Endo H to this substrate-affinity gel. After substrate-affinity chromatography, Endo H was separated from the coeluted glycopeptide substrate and some protein impurities by gel filtration and hydrophobic chromatography.

INTRODUCTION

Endo-*N*-acetyl-β-D-glucosaminidase H (Endo H) from *Streptomyces plicatus* releases *N*-linked high-mannose type oligosaccharide chains from glycoproteins by cleaving the β-D-(1→4) bond of the di-*N*-acetylchitobiosyl unit[1,2]. Thus, the enzyme is an important tool in glycoprotein research, as it can be used for recovery of both the oligosaccharides and the deglycosylated proteins[3]. Numerous applications of Endo H have been reported in the last few years. For instance, it was used in investigations of the function of carbohydrates in glycoenzyme stability and catalysis[4,5], and in elucidation of the biosynthetic pathway of glycoproteins in higher eucaryotes[6]. More recently, it was successfully applied to expose 2-acetamido-2-deoxy-D-glucose residues on yeast invertase, which could be then used as acceptor sites for galactosylation and subsequent sialylation by the corresponding transferases[7].

The enzyme is commercially available but rather expensive. Unfortunately, the current purification procedure[8] is quite time consuming, and the purification of

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Endo H may be simplified by introducing an affinity-chromatography step. Usually, affinity chromatography of an enzyme is performed with an immobilized inhibitor or cofactor[9]. When the enzyme does not require a cofactor and when inhibitors are not readily available, the substrate itself may be used as a ligand. For example, immobilized L-tryptophane methyl ester was able to bind alpha-chymotrypsin[10], and cellulases could be adsorbed onto amorphous cellulose at low temperature, and then released by a temperature shift[11]. Another example is the purification of amylase on immobilized glycogen[12]. In addition, endo-*N*-acetyl-α-D-galactosaminidase was purified on immobilized antifreeze glycoprotein with a salt- and temperature-induced elution[13].

We describe herein a procedure for the rapid purification of Endo H, as well as a simplified enzyme assay. A new type of substrate-affinity chromatography was introduced, in which elution of the enzyme took place under very mild conditions. As the solid support may be reused, this procedure appears well suited for the purification of larger quantities of Endo H.

EXPERIMENTAL

Materials. — *Streptomyces griseus* Pronase (EC 3.4.21.4 and 3.4.24.4) and lyophilized *Saccharomyces cerevisiae* invertase (EC 3.2.126) were from Boehringer Mannheim Biochemicals, and the latter was purified over DEAE-Sepharose[14]. Jack bean meal concanavalin A (Con A) was from Serva Fine Biochems. Gels and chemicals for electrophoresis were from Bio-Rad Laboratories, Sepharose and Sephadex gels were from Pharmacia Fine Chemicals, Inc., sugars were from Fluka Chem. Corp., and ^{3}H-labeled *N,N*-dimethylaminonaphthalenesulfonyl (dansyl) chloride (525 GBq/mmol) and Aquasol scintillation cocktail were from New England Nuclear.

Preparation of invertase glycopeptides. — Purified invertase (0.2 g) was digested with Pronase (20 mg) in 0.05M · Tris HCl, pH 7.5 (10 mL) and 5mM $CaCl_2$ for 16 h at 37°, followed by an additional incubation for 8 h with fresh Pronase (5 mg). After being boiled for 15 min, the sample was chromatographed on a Bio-Gel P-30 column (2.5 × 100 cm) in 0.1M acetic acid. The fractions comprising the two well-resolved glycopeptide peaks were concentrated by evaporation and dried in a desiccator over KOH pellets. Subsequently, they were analyzed for neutral[15] and for amino sugars[16] with D-mannose and 2-acetamido-2-deoxy-D-glucose as standards, respectively. The measured ratios of D-mannose to 2-acetamido-2-deoxy-D-glucose were 20 and 40 units to one, and they were named M20 and M40 glycopeptides, accordingly. [^{3}H]Dansylated M20 glycopeptides were prepared according to Tarentino and Maley[8]. The specific radioactivity was 3.1×10^7 d.p.m./μmol.

Enzyme assays. — Endo H activity was measured by a modification of the procedures as described[17,18]. Appropriately diluted enzyme was incubated in 50mM sodium acetate, pH 5.5 (0.1 mL) with ^{3}H-dansylated M20 glycopeptides (2×10^4 d.p.m.) for 15 min at 37°. The reaction was stopped by the addition of M Tris · HCl,

pH 8.5 (1 vol.), and boiling for 5 min. One fifth of the volume was mixed with Con A–Sepharose (1 mg.mL^{-1}; 0.2 mL) in 0.05M Tris·HCl buffer, pH 7.5, containing 0.5M NaCl, mM $CaCl_2$, and mM $MnCl_2$. The final volume was 0.4 mL and the suspension was incubated for 30 min with occasional inversion. The gel was centrifuged for 2 min in a microcentrifuge. The supernatant was removed by pipetting, combined with 0.2 mL of the washing fraction, and the radioactivity counted for tritium with Aquasol scintillation cocktail (10 mL) in a Beckman LS1800 counter using a d.p.m. data reduction program. The assay was performed in duplicate, and the activities were calculated from two incubations at different times using the boiled enzyme as a blank. The results of the assay were linear with time and proportional to the amount of enzyme until ~50% of the substrate was hydrolyzed. One unit of Endo H was defined as the amount of enzyme that hydrolyzes 1 μmol of M20 glycopeptides per min at 37°.

Exoglycosidases were assayed with the corresponding *p*-nitrophenyl glycosides at either pH 5.0 or 7.0. After incubation, the absorbance at 405 nm was measured for a solution in 0.1M Tris buffer, pH 7.2. The presence of proteases in the Endo H preparation was tested by the Azocoll–casein assay (Calbiochem) and by examination of the silver-stained sodium dodecyl sulfate gels for degradation products of invertase after incubation with Endo H (1 munit of Endo H, 1% Triton X-100, pH 5.2, 16 h at 37°). Proteins were determined with a Bio-Rad kit with bovine serum albumin as a standard.

Electrophoresis. — Proteins were analyzed by sodium dodecyl sulfate (SDS) electrophoresis in 0.7-mm thick poly(acrylamide) gels run in the discontinuous buffer system[19]. The gels were stained with silver[20].

Immobilizations of proteins. — Sepharose CL-4B was activated with 2,2,2-trifluoroethanesulfonyl (tresyl) chloride as described[21]. Partially purified Endo H (0.22 units, 5 U/mg) was coupled to activated Sepharose (25 mL) in 0.1M $NaH_2PO_4^-$–Na_2HPO_4 buffer, pH 7.0, for 15 h at 22°. As determined by activity mesurement of the immobilized enzyme, 4 mU of Endo H were immobilized per mL of gel. Con A was immobilized in 0.1M $NaH_2PO_4^-$–Na_2HPO_4 buffer, pH 8.0, and 0.5M NaCl to a level of 1–2 mg/mL, as determined by measurement of the absorbance at 280 nm before and after the coupling reaction (A_{280} = 1.14).

Estimation of the binding capacity of Con A–Sepharose for glycopeptides and Endo H. — The amount of invertase glycopeptides bound to Con A was determined in two ways. M40 glycopeptides (1.7 mg) were batchwise incubated with Con A–Sepharose (0.5 mL) for 3 h at 4° and pH 6.5. The gel was washed with the buffer (5 mL), and the glycopeptides were subsequently eluted with 0.5M methyl α-D-mannopyranoside. The latter fraction was concentrated by evaporation, and glycopeptides were separated from methyl α-D-mannopyranoside on a Bio-Gel P-4 column (1.5 × 50 cm). To determine the amount of glycopeptides bound to Con A–Sepharose, amino sugars were quantitatively determined in the glycopeptide fraction.

The binding capacity was also determined by a competition assay using a

modification of the method described[22]. [^{3}H]Dansylated M20 glycopeptides (0.24 nmol, 7800 d.p.m.) were bound to Con A–Sepharose (0.1 mL, 2 mg/mL) at pH 6.5 in a total volume of 0.25 mL. After equilibration for 30 min at 4° under stirring, the beads were sedimented in a minicentrifuge. Then, the radioactivity of 50 μL of the supernatant was counted for tritium and replaced with an equal volume of unlabeled M20 glycopeptides. The ratio of released-to-bound tritium was plotted against the amount of the competing glycopeptides added and the binding capacity was determined from the linear range of the curve. The pH dependence of glycopeptide binding to immobilized Con A was tested at pH 5.5, 6.5, and 7.5 by the same method. The buffers used were 50mM in sodium acetate, histidine·HCl, and Tris·HCl, respectively, and they all contained 0.5M NaCl, mM $CaCl_2$, and mM $MnCl_2$ (Con A buffers).

The binding of Endo H was tested as follows. Endo H from step III (0.1 unit, see below) was applied to Con A–Sepharose (2 mL) previously loaded with M40 glycopeptides at pH 5.5, 6.5, and 7.5 in the presence or absence of 0.5% Tween 20. The columns were washed with buffer (flow rate, 80 mL/h; 8 vols.) until no additional material absorbing at 280 nm was eluted. The unbound fractions were pooled, dialyzed against 0.05M sodium acetate, pH 5.5, and assayed for Endo H activity.

Purification of Endo H. — Step I. Culture filtrate. Streptomyces plicatus was grown batchwise in 10 L of a synthetic, chitin-based medium containing 0.4% of 2-acetamido-2-deoxy-D-glucose[23]. After 9 days of reciprocal shaking at 30°, Endo H activity did not increase anymore. Cells were then removed by filtration.

Steps II and III. Zinc acetate and ammonium sulfate precipitations were performed as described[8], except that the final dialysis was against 0.01M Tris·HCl, pH 7.5.

Step IV. Substrate-affinity chromatography. Con A–Sepharose (0.8 mg/mL; 100 mL) was equilibrated batchwise with M40 glycopeptides (150 mg) in the Con A buffer, pH 6.5, for 12 h. The unbound glycopeptides were removed with the same buffer (8 gel-vols.) on a sintered-glass funnel. Endo H from step III was equilibrated with the Con A buffer, pH 6.5, containing a few grains of phenylmethylsulfonyl fluoride. The solution was applied to the gel at 160 mL/h in a 5-cm wide chromatography column. The gel was washed with starting buffer at the same flow rate until the fractions were free from material absorbing at 280 nm. Endo H was eluted together with the glycopeptides with 0.5M methyl α-D-mannopyranoside (50 mL) in the same buffer. The column was then washed with the starting buffer (200 mL) at 40 mL/h. The fractions containing Endo H activity were concentrated to <1 mL in an ultrafiltration cell using a PM-10 membrane (Amicon), and further purified as given in step V. Unbound material could be concentrated and directly reapplied to the Con A–Sepharose column loaded with new glycopeptides. After several cycles, hydrolyzed glycopeptides in the unbound Endo H fraction may interfere with the binding of Endo H to the affinity column. Such a sample should then be either discarded or partially purified prior to application to the affinity gel.

Step V. Fast protein liquid chromatography (FPLC) on Superose 12B. Portions of 0.2 mL of concentrated Endo H sample were injected onto a Superose column and eluted at 0.25 mL/min in 0.05M sodium acetate, pH 5.5, containing 0.1M NaCl. This procedure was repeated with the remaining aliquots of the affinity-purified Endo H.

Step VI. Phenyl-Sepharose chromatography. Endo H from step V was applied to phenyl-Sepharose (2 mL) in 10% ammonium sulfate–0.05M sodium acetate, pH 5.2. The column was washed with the same buffer, and Endo H was subsequently eluted with 0.05M sodium acetate, pH 5.2. Fractions containing Endo H activity were dialyzed and frozen at −20°.

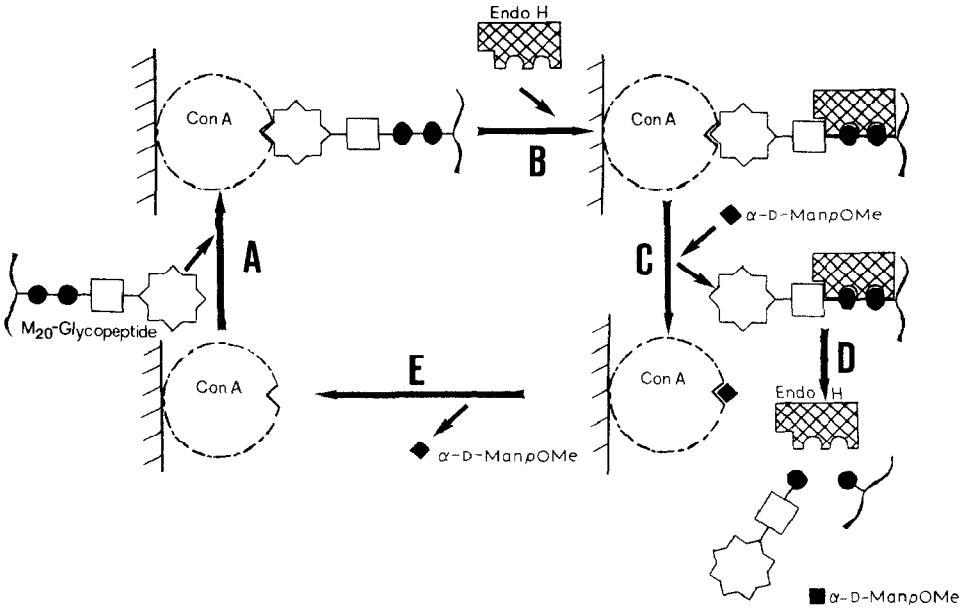
Endo H was also purified according to the procedure of Tarentino and Maley[8] with minor changes. Thus, culture filtrates were concentrated by ultrafiltration (Millipore), and DEAE-Sepharose and CM-Sepharose were used instead of the corresponding cellulose gels. The final step was a Bio-Gel P-30 gel filtration (column 2.5 × 100 cm) in 0.1M sodium acetate, pH 5.5.

RESULTS AND DISCUSSION

Initial attempts to purify Endo H by affinity chromatography. — As affinity ligands, we have tried to use $Man_{40}GlcNAc$ oligosaccharides and M40 glycopeptides. The former was produced by immobilized Endo H (see Experimental part). Both the oligosaccharides and the glycopeptides were coupled after periodate oxidation to Bio-Gel P-2-hydrazide[24]. Endo H was not retained at all by the first affinity gel, whereas from the second, ~80% of the applied activity was found in the effluent. Our attempts to elute the remaining 20% activity by competition with free glycopeptides, by change in pH, and by electrophoresis failed.

Substrate-affinity chromatography of Endo H. — From the aforementioned results, two points appeared crucial for the development of a successful Endo H affinity gel, *i.e.*, virtually intact glycopeptide substrate and a convenient way to elute the bound enzyme from the gel. Both requirements could be fulfilled by use of immobilized Con A to bind the glycopeptides which serve as a substrate for Endo H, and methyl α-D-mannopyranoside to elute these glycopeptides together with the bound enzyme (see Scheme 1).

Since the capacity of the affinity gel for Endo H depends on the amount of bound glycopeptides, the amount of invertase glycopeptides that can bind to Con A–Sepharose was determined. By amino sugar analysis and attribution of two GlcNAc residues per mole of glycopeptides[4], a capacity of ~0.1 μmol M40 glycopeptides per mL of gel at pH 6.5 was estimated. The binding capacity of Con A–Sepharose as a function of pH was determined by another method. At pH 5.5, 6.5, and 7.5, unlabeled glycopeptides were used to compete with [^{3}H]dansylated M20 glycopeptides previously bound to Con A–Sepharose. The curve was linear when 1 to 45% of the labeled glycopeptide was displaced (not shown). From this part of the curve, it was estimated that 1 mL of the affinity gel bound 77 nmol of glyco-



Scheme 1. (A) Con A–Sepharose is loaded with M20 glycopeptides consisting of two 2-acetamido-2-deoxy-D-glucose residues (●), the D-mannose core (□), and the outer chain (☆). (B) Endo H is bound to its substrate. (C) The enzyme-substrate complex is released from Con A–Sepharose by methyl α-D-mannopyranoside. The substrate is hydrolyzed both during and after this step (D). Con A–Sepharose is regenerated by extensive washing (E). The column can be loaded with fresh glycopeptides for a new purification cycle.

peptides. This roughly corresponds to the capacity determined by amino sugar measurement (see above). At all three pH values, the amount of bound glycopeptides was essentially the same. From the amount of protein immobilized, it seems that almost every Con A monomer can bind one glycopeptide molecule. Therefore, the upper limit of the gel capacity for Endo H should be ~80 nmol/mL. However, in practice, the capacity for Endo H is expected to be lower owing to continuous hydrolysis or reduced accessibility of the substrate (or both).

In order to find conditions where the enzyme binds to the affinity gel but does not hydrolyze the substrate too rapidly, the binding of Endo H was analyzed at different pH values in the presence or absence of 0.5% Tween 20. The range tested was determined by the pH-dependent activity and stability profile[8] of Endo H. At pH 6.5 without Tween 20, binding of Endo H was most favorable since only 26% of the applied activity remained unbound, compared to 32% at pH 7.5 and 65% at pH 5.5 (Table I). Since Con A bound the same amount of glycopeptides at all three pH values (see above), the optimal capacity at pH 6.5 probably results from a favorable balance between the binding strength of Endo H and its substrate-hydrolysis rate. At pH 5.5, Endo H activity is close to its maximum[8], and fast hydrolysis of the substrate most likely prevents good retention. The lower capacity at pH 7.5 (two units above the pH optimum) is probably caused by weaker binding of the enzyme to the substrate.

TABLE I

BINDING OF ENDO H TO THE SUBSTRATE-AFFINITY GEL AS A FUNCTION OF pH[a]

Activity	*pH*					
	5.5		*6.5*		*7.5*	
	Tween 20					
	−	+	−	+	−	+
Applied	100	100	100	100	100	100
Unbound	65	63	26	48	32	55

[a]The Con A–Sepharose column (2 mL; 1 mg of protein/mL) was loaded with M40 glycopeptides at the indicated pH. Endo H from step IV (0.1 unit) was applied in the presence or absence of 0.5% Tween 20 at 4°. The columns were washed with the starting buffers, and the activity of unbound Endo H was determined as described in the Experimental section.

To confirm that Endo H retention was not due to nonspecific interactions with Con A or Sepharose, partially purified Endo H (0.2 units) was applied to pure Con A–Sepharose (2 mL) at pH 6.5. Over 90% of the activity could be recovered by washing the column with buffer (not shown), demonstrating that Endo H retention in the affinity column was due to specific interaction with its substrate.

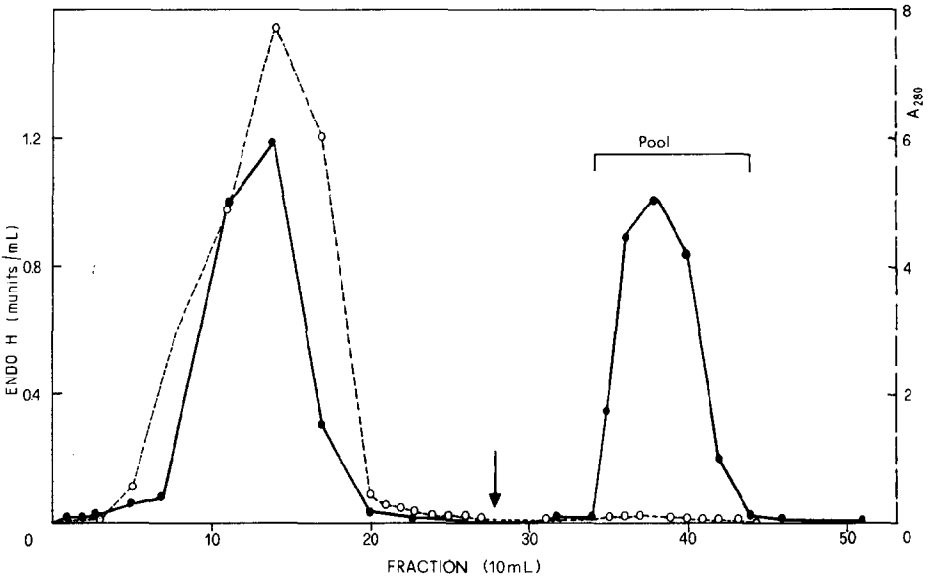


Fig. 1. Substrate affinity chromatography of Endo H. Endo H (3.0 units) was applied to Con A–Sepharose column (100 mL) loaded with invertase glycopeptides. The unbound proteins were washed with the starting buffer, and Endo H was eluted with 0.5M methyl α-D-mannopyranoside. For additional details see the Experimental section. The full line indicates Endo H activity and the dashed line absorbance at 280 nm. The arrow designates a change from the starting buffer to 0.5M methyl α-D-mannopyranoside. The values shown for the Endo H activity in the second peak are somewhat underestimated owing to competition of the labeled with the unlabeled glycopeptides that were present in these fractions.

TABLE II

PURIFICATION OF ENDO *N*-ACETYL-β-D-GLUCOSAMINIDASE H FROM *Streptomyces plicatus*

Purification step	*Batch*	*Volume (mL)*	*Activity (units)*	*Protein (mg)*	*Specific activity units/mg*	*Over-all yield (%)*	*Yield per step (%)*	*Purification factor*
I Culture filtrate	1	8000	8.0	1200	0.0066	100	100	1
	2	4750	3.9	760	0.0051	100	100	1
II Zinc precipitation	1	1670	4.2	317	0.0121	52	52	1.8
	2	1030	1.74	206	0.0079	45	45	1.5
III Ammonium sulfate (0.4–0.9)	1	158	3.0	159	0.0190	37	71	2.9
precipitation	2	45	1.3	95	0.0137	33	73	2.7
IV Substrate-affinity	1[a]	0.6	1.0[b]	0.054	18.4[b]	12.5[b]	34	2800
	2[a]	0.8	0.67[b]	0.042	16.0[b]	17.2[b]	52	3100
V FPLC Superose 12B	1+2[c]	3.0	1.4	0.069	20.3	11.8	84	3400

[a]Con A–Sepharose (100 mL; 0.8 mg/mL). [b]Activity of bound Endo H is expressed as a difference between the applied and unbound activities. [c]Pooled batches 1 and 2 from step IV.

Intact invertase bound to Con–A Sepharose was also tested for substrate-affinity chromatography of Endo H. Endo H was indeed bound, and it could be released by methyl α-D-mannopyranoside from the invertase–Con A column. However, after SDS electrophresis, several other *S. plicatus* proteins were detected in the fraction eluted with methyl α-D-mannopyranoside, indicating that intact invertase has lower selectivity for Endo H. As expected, eluted invertase was partially deglycosylated (data not shown).

A larger amount of *S. plicatus* proteins, obtained by zinc acetate and ammonium sulfate precipitation, was applied to a 100-mL column containing Con A–Sepharose loaded with glycopeptides. The results (Fig. 1 and Table II) showed that a high enrichment of Endo H was achieved by the application of substrate-affinity chromatography. Endo H activity was eluted together with the glycopeptides in a broad peak. It was difficult to determine quantitatively the activity of Endo H in this fraction, because the coeluted glycopeptides competed with the [^{3}H]dansylated glycopeptides in the enzyme assay. Assuming that no enzyme remained on the gel, it was estimated, on the basis of the applied and unbound activities, that 30–50% of the enzyme bound to the affinity gel. This assumption is reasonable, since the glycopeptides were quantitatively eluted by methyl α-D-mannopyranoside (unpublished results), and Con–A Sepharose alone does not bind Endo H. In one affinity-chromatography cycle, ~50 μg of Endo H could be obtained with 100 mL

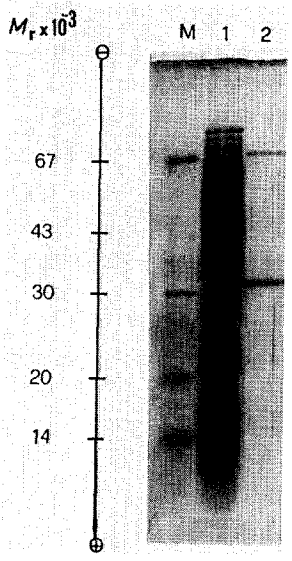


Fig. 2. Analysis of the purified Endo H by sodium dodecyl sulfate electrophoresis. Proteins from the culture filtrate of *S. plicatus* after zinc acetate and ammonium sulfate precipitations (15 μg) were applied to lane 1. Endo H obtained after FPLC gel filtration (0.2 μg) was electrophorezed in lane 2. The standard proteins were bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 000), and α-lactalbumin (14 000). The gel contained 12% acrylamide (2% C) and the experiment was performed in the discontinuous buffer system[19].

of the affinity gel. The enzyme was purified ~1000-fold in this step, and its specific activity increased to 16–18 units/mg.

However, Endo H was not pure after substrate-affinity chromatography. Such a preparation still contained glycopeptides and methyl α-D-mannopyranoside, as well as a few proteins from *S. plicatus*. The enzyme was therefore concentrated by ultrafiltration and subjected to gel filtration on a Superose 12B column. Endo H activity was eluted in a sharp peak, which was followed by a broad sugar peak consisting of oligosaccharides and methyl α-D-mannopyranoside (not shown). In the pooled Endo H fraction, the presence of neutral sugars could barely be detected by the phenol–sulfuric acid assay. The specific activity of Endo H increased to 20 units/mg. Most of the contaminating proteins were removed in this step, except one major protein with M_r ~66 kDa and traces of a few others (Fig. 2). These proteins had no detectable protease or glycosidase activities. Therefore, this Endo H preparation could be used for deglycosylation of glycoproteins. However, if necessary, the contaminating proteins can be removed by hydrophobic chromatography on phenyl-Sepharose under the conditions described in the Experimental section. After this step, Endo H had a specific activity of 27 units/mg.

The molecular mass of Endo H purified by substrate affinity chromatography was ~31 kDa (Fig. 2). This is close to the value reported for the conventionally purified enzyme[8]. Such an enzyme had a specific activity[2] of 38–40 units/mg, which is higher than the specific activity of the substrate-affinity chromatography purified Endo H. However, the difference might be due, at least in part, to the different substrate and the modified assay system, which we used. Our assay was based on Con A–Sepharose which was added to the reaction mixture to bind the remaining substrate and oligosaccharide product. The amount of the other, ^{3}H-labeled product was then determined. The assay was reproducible (standard deviation <5%) except for Endo H fractions containing glycopeptides from the substrate-affinity column (see above). Some difficulties were reportedly encountered when measuring Endo H activity in crude samples[25], but we have found the assay described herein to also be reliable during the purification of Endo H by conventional methods.

The purified enzyme was stored at −20° in 0.1M sodium acetate, pH 5.2. It could be thawed and refrozen at least five times without any loss of the enzymic activity. Therefore, it was not necessary to add a stabilizing protein like bovine serum albumin as in some commercial Endo H preparations.

The substrate-affinity chromatography described herein differs in several aspects from the substrate-affinity-chromatography procedures described thus far. Since the substrate is not covalently linked to the matrix, very mild conditions can be used to elute the bound enzyme together with its substrate. In addition, the matrix can be reused after loading with fresh substrate. Moreover, this approach should also simplify the purification of other high-mannose-type endoglycosidases, which have thus far been isolated from bacteria, fungi, and animal cells[26–28].

Since it requires only an oligosaccharide substrate and a lectin, we believe

that the substrate-affinity chromatography described herein could be used for purification of endoglycosidases and *N*-glycanases specific for complex-type glycans. This will make the method even more valuable, as these enzymes are widely used in various aspects of glycoconjugate research.

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