Purification and Properties of an Endo-β-N-acetylglucosaminidase from Streptomyces griseus

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SUMMARY

An enzyme that hydrolyzes di-N-acetylmaltobiose linkages in oligosaccharides and glycoproteins was purified to homogeneity from cultural filtrates of Streptomyces griseus. The molecular weight of the enzyme, determined by sedimentation equilibrium analysis, is 27,200 ± 200, and it appears to consist of a single polypeptide chain. This apparent endo-β-N-acetylglucosaminidase was completely stable at 37° for at least 48 hours and resistant to inactivation by several proteolytic enzymes. Treatment of Asn-(GlcNAc)₂(Man)₆ with the enzyme yielded Asn-GlcNAc + (GlcNAc)₂(Man)₆(GlcNAc)₂, and GlcNAc + (GlcNAc)(Man)₆(GlcNAc)₂, respectively. The [³H]dansyl-Asn derivatives, which were rapidly hydrolyzed also, were used as substrates to assay the enzyme. Similar findings obtained with specific glycoproteins are described in the accompanying paper (TARENTINO, A. L., PLUMMER, T. H., JR., AND MALEY, F. (1974) J. Biol. Chem. 249, 818-824). Asn-(GlcNAc)(Man)₆ was not hydrolyzed by this enzyme but was hydrolyzed by another apparent endo-β-N-acetylglucosaminidase. The latter could be separated from the Asn-(GlcNAc)(Man)₆ hydrolyzing endoglycosidase by Sephadex G-100 chromatography.

The "core" glycosyl asparagine derivative, Asn-(GlcNAc)(Man)₆ (Man)₆, used previously (1) consists of at least two enzymes, one specific for high, the other for low molecular weight oligosaccharides. We shall refer to these as endo-β-N-acetylglucosaminidases H and L, respectively.

This report is concerned primarily with the purification to homogeneity of endoglycosidase H and the nature of some of its properties. The following paper describes the potential utility of this enzyme in clarifying glycoprotein structure and function.

EXPERIMENTAL PROCEDURE

Materials

Ovalbumin (Grade V) and chitin were purchased from the Sigma Chemical Co. Pronase (B grade), Azocoll, and Azure blue B were obtained from Calbiochem. Dried Micrococcus lysodeikticus cell walls were purchased from Worthington Biochemical Co., dansyl chloride from Pierce Chemical Co., and [³H]dansyl chloride (specific activity, 1.25 Ci per mmole) from the New England Nuclear Corp. Protein solutions were concentrated by ultrafiltration with an Amicon apparatus (Amicon Corp.).

DEAE-cellulose and CM-cellulose (microgranular advanced ion exchange celluloses), obtained from Reeve Angel Co., were precycled, equilibrated with the appropriate buffer, and packed into columns under a pressure of 10 psi (30% slurry). Sulfoethyl Sephadex C-25 and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Inc.

Methods

Preparation of Ovalbumin Asparagine Oligosaccharides—Ovalbumin glycopeptide was prepared by pronase digestion of ovalbumin by methods described previously (2). The glycopeptide was digested further with bovine carboxypeptidase A as described by Kimmel and Plummer (3), and the resulting heterogeneous asparagine-oligosaccharide mixture was separated into homogeneous fractions by the procedure of Huang et al. (4). The fraction containing asparagine, N-acetylglucosamine, and mannose in the ratio of 1:4:6 (6.5 pmoles) was dansylated (5) and purified by chromatography on Sephadex G-25 (1.5 x 98 cm) with 0.1 M acetic acid. The product, dansyl-Asn-(GlcNAc)₆(Man)₆, was located by its strong yellow-orange fluorescence on exposure of the tubes to ultraviolet light and its lack of reactivity with ninhydrin. The phenolsulfuric assay (6) was used to quantitate this material.

The oligosaccharide fraction containing Asn-(GlcNAc)₆(Man)₆ was digested with jack bean α-D-mannosidase to Asn-(GlcNAc)₆(Man)₆. The "core" glycosyl asparagine derivative, Asn-(GlcNAc)₆(Man)₆ (Man)₆ from RNase B and ovalbumin, was characterized previously (1) with the aid of an endoglycosidase partially purified from the cultural filtrates of Streptomyces griseus. The molecular weight of the enzyme, determined by sedimentation equilibrium analysis, is 27,200 ± 200, and it appears to consist of a single polypeptide chain. This apparent endo-β-N-acetylglucosaminidase was completely stable at 37° for at least 48 hours and resistant to inactivation by several proteolytic enzymes. Treatment of Asn-(GlcNAc)₆(Man)₆ with the enzyme yielded Asn-GlcNAc + (GlcNAc)₂(Man)₆(GlcNAc)₂, and GlcNAc + (GlcNAc)(Man)₆(GlcNAc)₂, respectively. The [³H]dansyl-Asn derivatives, which were rapidly hydrolyzed also, were used as substrates to assay the enzyme. Similar findings obtained with specific glycoproteins are described in the accompanying paper (TARENTINO, A. L., PLUMMER, T. H., JR., AND MALEY, F. (1974) J. Biol. Chem. 249, 818-824). Asn-(GlcNAc)(Man)₆ was not hydrolyzed by this enzyme but was hydrolyzed by another apparent endo-β-N-acetylglucosaminidase. The latter could be separated from the Asn-(GlcNAc)(Man)₆ hydrolyzing endoglycosidase by Sephadex G-100 chromatography.

1 The abbreviations used are: Asn-(GlcNAc)₆(Man)₆, 2-ace taminido-4-O-α-mannopyranosyl (at → 4).2-ace taminido-2-deoxy-β-D-glucopyranosyl)₆(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; Asn-GlcNAc, 2-ace taminido-N-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine; and dansyl chloride, 5-dimethylamino-naphthalene-1-sulphonyl chloride.
Instruments, Inc.). To detect enzyme activity, the gel slices were incubated in 0.5 ml of 0.5 M sodium citrate, pH 5.5, at electrophoretically. The other half was frozen in Dry Ice and at 4°C for 50 min. The gels were extruded and sliced lengthwise with a razor blade. One-half of each gel was stained for protein using a modification of the Davis procedure (12). The protein sample (100 µg) was layered on top of the polymerized gels in 0.1 ml of 20% sucrose and electrophoresed (2 mA per gel) cut into 1-mm sections with a Mickle gel slicer (Brinkmann Instruments, Inc.). To detect enzyme activity, the gel slices were incubated in 0.5 ml of 0.5 M sodium citrate, pH 5.5, at 37°C for 30 min, and a 5-µl aliquot of each incubation mixture was removed for assay as described above.

Amino Acid Analysis—Amino acid analysis was performed by the method of Spackman et al. (13) with a JEOL Co. model 5AII amino acid analyser. For sulphhydril analysis, 890 µg of enzyme protein were hydrolyzed in 6 M HCl containing 0.2 M dimethylsulfoxide as described by Shapiro et al. (15).

Molecular Weight Determinations

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—Molecular weight determinations in sodium dodecyl sulfate acrylamide gels were performed by a method similar to that described by Shapiro et al. (15).

The gels were stained for 2 hours in a solution of 0.2% Coomassie blue in 45% methanol-9% acetic acid and then destained electrophoretically in 7.5% acetic acid-6% methanol (16). Mobility was calculated as the ratio of protein migration distance to that of gel length and plotted against the log of molecular weight.

Sephadex G-100 Chromatography—Proteins of known molecular weight were used to calibrate a column of Sephadex G-100 (1.5 × 220 cm) that had been equilibrated previously with a solution of 0.01 M potassium phosphate, pH 8.45, containing 0.1 M sodium chloride. Samples were placed on the column in 1.0 ml and eluted at a flow rate of 5.4 ml per hour. Fractions of 3 ml were collected.

The void volume (V0) of the column (135 ml) was determined with immunoglobulin G, and the elution volume (Ve) was approximated from the midpoint of the eluted protein or enzyme peaks.

Ultracentrifuge Analysis—Sedimentation equilibrium analysis was performed by the meniscus depletion method of Yphantis (17) using a Spinco model E ultracentrifuge equipped with a photoelectric scanner. Molecular weight (Mw) was calculated from the equation

$$M_w = \frac{2RT}{(1 - \bar{v})\bar{v}c} \cdot \frac{dn}{d\bar{v}}$$

where the partial specific volume, \(\bar{v}\), was determined from the amino acid composition (18) and found to be 0.725 ml per g. The concentration, c, which is proportional to recorder deflection, and the distance from the axis of rotation, r, were used to calculate the slope dnc/dr. The protein concentration was 250 µg per ml. Molecular weight determinations were not extrapolated to zero protein concentration.

Spectral Studies—Circular dichroism spectra were obtained with a Cary model 61 spectropolarimeter using 0.5-mm cylindrical quartz cells and constant nitrogen purging. Spectra were scanned at 3 nm per min with a pen period of 3 s. Fluctuations in the recorder pen at the peak height were no greater than 0.03%. The data are reported as mean residue ellipticity (deg cm² per dmole), which is given by the equation:

$$\theta = \frac{\theta^\circ M}{10^c}$$

where M is the mean residue molecular weight determined as 108 from the amino acid analysis and molecular weight, \(\theta^\circ\) is the observed ellipticity in degrees, l is the cell path length in cm, and c is the protein concentration in g per cm³. Ultraviolet measurements were made with a Cary model 14 recording spectrophotometer.
Purification of Endo-β-N-acetylglucosaminidase H

**Step 1: Cultural Filtrate**—A strain of *S. griseus* (ATCC No. 27800) was obtained from the Worthington Biochemical Co. and maintained on Sabouraud dextrose agar slants. For enzyme production, the stocks were transferred to plates of Hickey-Tresner sporulating agar (19) and incubated at 25°C for a minimum of 1 week. The spores were removed from the plates with 10 ml of sterile 1% Triton X-100 and added to 1 liter of Jeuniaux's medium (9). After 3 days of growth in a New Brunswick rotary shaker at 30°C, 50-ml aliquots were transferred to 12 2-liter flasks each containing 1 liter of the same medium, and the growth process was repeated for another 3 days. At the end of this period, the endo-β-N-acetylglucosaminidase H activity reached a plateau of about 0.005 unit per ml of culture fluid, and the cultures were harvested by filtration through Schleicher and Schuell 520B 1/4 paper. About 10 liters of filtrate were obtained containing 1 mg per ml of protein.

**Step 2: Zinc Precipitation**—The cultural filtrate (10 liters) was raised to 0.075 M Zn²⁺ by the dropwise addition, with continuous stirring, of 75 ml of 1 M zinc acetate per liter at 25°C. Solid Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) base was added periodically to maintain the pH at 7.0. The resulting precipitate was allowed to settle for at least 2 hours at 25°C and then overnight at 4°C. The latter temperature was used throughout the following procedures. The clear supernatant was siphoned off and the remaining suspension was centrifuged at 4000 x g for 10 min to yield a gelatinous pellet, which was dissolved in 0.3 M sodium citrate, pH 5.5.

**Step 3: Ammonium Sulfate Precipitation**—The extract from Step 2 (1,700 ml containing 4 mg per ml of protein) was brought to 40% saturation by the addition of 22.5 g of solid ammonium sulfate per 100 ml of solution. The mixture was stirred for 45 min and centrifuged at 13,000 x g for 15 min. The supernatant fraction was adjusted to 90% saturation by the addition of 33.5 g of solid ammonium sulfate per 100 ml of solution, stirred for 45 min, and centrifuged as described above. The precipitate was dissolved in a minimal volume of 0.01 M potassium phosphate, pH 8.45, and dialyzed for 3 days against four 6-liter changes of the same buffer.

**Step 4: DEAE-cellulose Chromatography**—The dialyzed extracts from three of the above preparations were pooled (696 ml containing 4.95 g of protein) and applied to a DEAE-cellulose column (3.7 x 33 cm) at a flow rate of 100 ml per hour. The column, equilibrated previously with 0.01 M potassium phosphate, pH 8.45, was washed with this buffer at 75 ml per hour until the unabsorbed protein was eluted. Elution was continued with a linear gradient, starting with 1.5 liters of 0.01 M potassium phosphate, pH 8.45, in the mixing chamber and 1.5 liters of 0.32 M potassium phosphate, pH 8.45, in the reservoir (Fig. 1). Fractions of 20 ml were collected. The endo-β-N-acetylglucosaminidase H-containing fractions (66 to 85) were pooled and lyophilized. The residue was dissolved in a minimal volume of 0.01 M sodium acetate, pH 4.85, and dialyzed for 3 days against three 6-liter changes of the same buffer.

**Separation of Endo-β-N-acetylglucosaminidase H and L—** Fractions 86 to 115, which contained the trailing edge of the endo-β-N-acetylglucosaminidase H activity and most of the endo-β-N-acetylglucosaminidase L were pooled, lyophilized, and chromatographed on Sephadex G-100 as described below. As shown in Fig. 2, the two enzyme activities eluted in separate regions with endo-β-N-acetylglucosaminidase L appearing to be larger in molecular weight than H.
Step 5: Sulfathyl Sephadex Chromatography—The dialyzed extract from Step 4 (114 ml containing 260 mg of protein) was applied at a flow rate of 36 ml per hour to a column of sulfathyl Sephadex C-25 (2 × 40 cm) which had been equilibrated previously with 0.01 M sodium acetate, pH 4.85. The column was washed with acetate buffer until the unabsorbed protein was eluted. It was then developed with a linear gradient starting with 800 ml of 0.01 M sodium acetate, pH 4.85, in the mixing chamber and 800 ml of 0.10 M sodium acetate, pH 4.85, in the reservoir (Fig. 3). Fractions of 9 ml were collected at a flow rate of 36 ml per hour. The enzyme-containing fractions (78 to 98) were pooled and lyophilized, and the residue was dissolved in 5.5 ml of 0.01 M potassium phosphate, pH 8.45.

Step 6: Sephadex G-100 Chromatography—The enzyme preparation from Step 5 (5.5 ml containing 11 mg of protein) was applied to a column of Sephadex G-100 (1.5 × 220 cm) which had been equilibrated previously with 0.01 M potassium phosphate, pH 8.45. The column was developed with the same buffer at a flow rate of 5 ml per hour and 3-ml fractions were collected (Fig. 4). The enzyme-containing fractions (84 to 94) were pooled, dialyzed against 0.01 M ammonium acetate, pH 4.5. The enzyme emerged sharply from the column at pH 4.8, with the specific activity essentially constant across the elution peak. The fractions containing the enzyme were pooled, dialyzed against 0.01 M potassium phosphate, pH 4.5, and concentrated by ultrafiltration to 5 ml. The enzyme can be stored at 4°C for at least 6 months under these conditions with no loss in activity.

Sources of Endo-β-N-acetylglucosaminidase H—This glycosidase appears distinct from the chitinase components elaborated by S. griseus since it did not chromatograph with any of the known chitinase activities on either DEAE-cellulose or Sephadex G-100 and whereas the endoglycosidase varied appreciably in the cultural fluids from different strains, chitinase did not. In addition, the endoglycosidase did not vary greatly when the cells were grown on glucose, but the chitinase activity was reduced considerably. At present, the enzyme has been found mainly in some plant and bacterial sources, but has not been clearly demonstrated in animal tissues. Cultural extracts from Diplococcus pneumoniae appear to contain a similar, if not identical, enzyme (20). The utility of this enzyme to the organisms that elaborate it is not apparent at this time.

**RESULTS AND DISCUSSION**

**Enzyme Purity**—As shown in Table I, S. griseus endo-β-N-acetylglucosaminidase H was purified 4000-fold from the cultural filtrate with a 38% recovery of activity. A single sharp protein band which migrated in the same region as the enzyme activity (Fig. 5) was obtained on polyacrylamide disc gel electrophoresis of the Step 7 enzyme. The final enzyme preparation was devoid of exo-β-N-acetylglucosaminidase, chitobiase, α- and β-D-mannosidase, and glycosyl asparaginase activities. It was also protease-free as determined by Azocoll and Azur blue B digestions. No hydrolysis of chitin, chito-oligosaccharides, or M. lysodeikticus cell walls was observed, even on prolonged incubation.

**Hydrolysis of Glycosyl Asparagine Derivatives**—We have previously indicated (1) that commercial chitinase contains endoglycosidase(s) directed against the internal N-acetylglucosamine residues of either Asn-(GlcNAc)_{2n}(Man)_{p} and Asn-(GlcNAc)_{n}(Man)_{p}. On re-examination of the substrate specificity of the pure endoglycosidase (endo-β-N-acetylglucosaminidase H), it was found that while Asn-(GlcNAc)_{n}(Man)_{p} and its dansyl derivative were equally effective substrates, neither Asn-(GlcNAc)_{n}(Man)_{p} nor its dansyl derivative was hydrolyzed (Table II). Since these initial studies, we have found that Asn-(GlcNAc)_{n}(Man)_{p} is hydrolyzed by another enzyme which copurifies with endo-β-N-acetylglucosaminidase H through Step 3 of the purification procedure. At Step 4, however, the enzyme that hydrolyzes dansyl-Asn-(GlcNAc)_{n}(Man)_{p} could be distinguished from that which hydrolyzes dansyl-Asn-(GlcNAc)_{n}(Man)_{p}, since the former enzyme eluted sharply between tubes 66 and 85 on DEAE-cellulose (Fig. 1), whereas the latter partially overlapped this region but eluted mainly between tubes 86 and 115 (not shown). A complete separation of the two enzymes could be effected on Sephadex G-100 (Fig. 2). Endo-β-N-acetylglucosaminidase L does not appear related to the chitobiase activity in cultural filtrates of S. griseus (9, 22), since it does not hydrolyze di-N-acetylchitobiose.

As indicated in Table II, removal of the asparagine moiety

<table>
<thead>
<tr>
<th>Step</th>
<th>Purification step</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cultural filtrate</td>
<td>31,000</td>
<td>162</td>
<td>33,100</td>
<td>0.0049</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>Zinc precipitation</td>
<td>5,150</td>
<td>134</td>
<td>10,300</td>
<td>0.0089</td>
<td>83</td>
</tr>
<tr>
<td>3.</td>
<td>Ammonium sulfate</td>
<td>5,600</td>
<td>123</td>
<td>4,950</td>
<td>0.0249</td>
<td>76</td>
</tr>
<tr>
<td>4.</td>
<td>DEAE-cellulose (pH 8.45)</td>
<td>325</td>
<td>133</td>
<td>260</td>
<td>0.512</td>
<td>82</td>
</tr>
<tr>
<td>5.</td>
<td>SE-Sephadex (pH 4.85)</td>
<td>169</td>
<td>92</td>
<td>11</td>
<td>8.4</td>
<td>57</td>
</tr>
<tr>
<td>6.</td>
<td>Sephadex G-100</td>
<td>33</td>
<td>88</td>
<td>4.7</td>
<td>19.0</td>
<td>54</td>
</tr>
<tr>
<td>7.</td>
<td>CM-cellulose (pH 4.5)</td>
<td>18</td>
<td>62</td>
<td>2.7</td>
<td>23.0</td>
<td>38</td>
</tr>
</tbody>
</table>

*Steps 1 through 3 were worked up in separate 10-liter lots and combined for Step 4.*

**Fig. 5.** Polyacrylamide disc gel electrophoresis of Streptomyces griseus endo-β-N-acetylglucosaminidase H (specific activity, 23). For details, see "Methods."
from Asn-(GlcNAc)₆(Man)₂(GlcNAc)₂ with glycosyl asparaginase or addition of a dansyl group to the asparagine did not affect the endoglycosidase H hydrolysis rate to any great degree. Length, however, appears to contribute to the substrate specificity, for whereas Asn-(GlcNAc)₆(Man)₂ was hydrolyzed at a rate comparable to that of larger oligosaccharides (Table II), the hydrolysis of Asn-(GlcNAc)₆(Man)₂ could not be detected. Another limiting factor appears to be the environment surrounding the oligosaccharide, for as shown in the following paper, susceptible proteins are relieved of their associated oligosaccharides at different rates. In the case of proteins with more than one oligosaccharide unit, the enzyme hydrolyzes some chains and not others, although they all contain a di-N-acetylchitobiose core. Specificity studies are limited at present, however, by the lack of a broad spectrum of oligosaccharides containing di-N-acetylchitobiose.

**pH Optima and Enzyme Stability**—With dansyl-Asn-(GlcNAc)₄(Man)₄ as substrate, the enzyme showed maximal activity between pH 5.0 and 6.0 (Fig. 6), but no hydrolysis of dansyl-Asn-(GlcNAc)₄(Man)₄ could be detected at any pH tested. From initial velocity measurements at pH 5.5, an apparent Kₘ of 0.3 mM was obtained for dansyl-Asn-(GlcNAc)₄(Man)₄.

The purified enzyme did not lose activity on repeated freezing and thawing or lyophilization and lost activity rapidly when incubated below pH 4.5 at 37°C (Fig. 6). From pH 4.5 to 8.5, however, dilutions of enzyme containing as little as 0.3 μg of protein per ml were completely stable during incubation at 37°C for 48 hours. The stability of this enzyme is illustrated further by the finding that pronase, trypsin, and chymotrypsin at concentrations as high as 2.5 mg per ml, when incubated with 5 to 10 μg of endoglycosidase, had little or no effect on enzyme activity over a 5-hour period at 37°C. However, pronase at 5 mg per ml inactivated the enzyme slowly during a 12-hour period. This remarkable stability is extremely useful where prolonged incubations are required.

**Effect of Potential Inhibitors**—The following compounds had little or no effect on enzyme activity when assayed either directly (as described under "Methods") or with prior incubation of the enzyme: Asn-GlcNAc, 1 mM; Asn-(GlcNAc)(Man); 2 mM; (Man)₄(GlcNAc)₂, 0.8 mM; di-N-acetylchitobiose, 2 mM; tri-N-acetylchitotriose, 2 mM. Preincubation of the enzyme for 1 hour in the presence of either 1 mM HgCl₂ or chloromercuri-

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**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydrolysis rate (μmoles/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)₄</td>
<td>0</td>
</tr>
<tr>
<td>Asn-GlcNAc₆(Man)₄</td>
<td>0</td>
</tr>
<tr>
<td>dansyl-Asn-(GlcNAc)₄(Man)₄</td>
<td>0</td>
</tr>
<tr>
<td>Asn-(GlcNAc)₆(Man)₂</td>
<td>29.0</td>
</tr>
<tr>
<td>Asn-(GlcNAc)₆(Man)₂</td>
<td>22.5</td>
</tr>
<tr>
<td>dansyl-Asn-(GlcNAc)₆(Man)₂(GlcNAc)₂</td>
<td>16.3</td>
</tr>
<tr>
<td>(GlcNAc)₆</td>
<td>18.0</td>
</tr>
</tbody>
</table>

*a* Assayed colorimetrically by release of N-acetylglucosamine (21).

*b* Assayed by glycosyl asparagine analysis (3).

*c* Dansyl compounds were assayed by the radioactive procedure (see "Methods").

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**Table III**

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Residues per 27,200 gᵦ</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>34.0</td>
<td>34</td>
</tr>
<tr>
<td>Threonine</td>
<td>13.4b</td>
<td>13</td>
</tr>
<tr>
<td>Serine</td>
<td>16.1b</td>
<td>16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.7</td>
<td>21</td>
</tr>
<tr>
<td>Proline</td>
<td>11.7</td>
<td>12</td>
</tr>
<tr>
<td>Glycine</td>
<td>24.2</td>
<td>24</td>
</tr>
<tr>
<td>Alanine</td>
<td>28.2</td>
<td>28</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.02</td>
<td>1</td>
</tr>
<tr>
<td>Valine</td>
<td>21.2b</td>
<td>21</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.72</td>
<td>3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.81b</td>
<td>9</td>
</tr>
<tr>
<td>Leucine</td>
<td>17.9</td>
<td>18</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>13.7</td>
<td>14</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>9.57</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.5</td>
<td>12</td>
</tr>
<tr>
<td>Histidine</td>
<td>5.55</td>
<td>6</td>
</tr>
<tr>
<td>Arginine</td>
<td>12.4</td>
<td>12</td>
</tr>
</tbody>
</table>

*Unless otherwise indicated, these values represent an average of those obtained at 24, 48, and 72 hours.

*b* Extrapolated to zero hydrolysis time.

*b* Determined as cysteic acid (14).

*b* Taken from the 72-hour value.
acids glycine, alanine, valine, leucine, and isoleucine. The protein contains 3 residues of methionine and no more than 1 residue of half-cystine. The latter determination remains questionable because of the lack of sufficient material for large scale amino acid analyses. However, the results with HgCl$_2$ and chloromercuribenzoate suggest, but do not prove, the presence of a sulfhydryl group. The endo-$\beta$-N-acetylglucosaminidase does not appear to be a glycoprotein since glucosamine was not evident in the amino acid analyses and the protein was phenolsulfuric acid-negative.

Molecular Weight Determinations—Sedimentation equilibrium analysis of the enzyme gave linear plots of ln absorbance versus $r^2$, as expected for a homogeneous protein (Fig. 7). The molecular weight of the enzyme calculated from six independent runs was 27,200 $\pm$ 200. This value is in excellent agreement with a molecular weight of 28,000 calculated by sodium dodecyl sulfate acrylamide gel electrophoresis (Fig. 8). A somewhat lower value of 23,500 was obtained by Sephadex G-100 chromatography (Fig. 8).

Spectral Properties—In view of the unusual stability of the enzyme to conditions that are often detrimental to protein structure (prolonged incubation at elevated temperatures, freeze thawing, proteolytic digestion), it was considered possible that this feature might be reflected in the spectral properties of the endoglycosidase. The ultraviolet spectral analysis (Fig. 9, top) revealed a double absorption peak at 278 and 282 nm, with a shoulder in the 290 to 295-nm region. The latter is no doubt indicative of tryptophan, and the former two are probably due to tyrosine, but the presence of a double peak is somewhat unusual. The spectrum is unchanged in 6 M guanidine hydrochloride and is similar to that of pancreatic ribonuclease, except for the absence of a 295-nm band in the latter enzyme (23).

CD analysis (Fig. 9, bottom) revealed a major ellipticity band at 220 nm, which probably represents a major contribution of $\beta$ structure to the enzyme, although this band is at a slightly longer wavelength than the 217 nm usually given (24). As expected, both the CD spectrum and the enzyme activity were abolished in 6 M guanidine hydrochloride. On extensive dialysis, however, about one-half of the activity was restored, but the original spectrum was not, as a broad band at 220 nm with a shoulder at 208 nm was now apparent.

Acknowledgments—We wish to thank Georgina Evans for her able assistance during the course of much of this work and Dr. Robert MacColl for the sedimentation equilibrium analyses. We would also like to express our appreciation to Dr. Thomas H. Plummer, Jr., for his aid with some of the substrate specificity studies and to Dr. Gladys F. Maley for helpful suggestions.

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Purification and Properties of an Endo-β-N-acetylglucosaminidase from 
*Streptomyces griseus*

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