THE USE OF ENDO-β-N-ACETYLGLUCOSAMINIDASE H IN CHARACTERIZING THE STRUCTURE AND FUNCTION OF GLYCOPROTEINS

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SUMMARY

Endo-β-N-acetylglucosaminidase H from Streptomyces plicatus can be useful in determining both the molecular weight of the protein moiety of glycoproteins and their inherent number of oligosaccharide chains. In the case of carboxypeptidase Y the molecular mass of the carbohydrate free protein was confirmed as 51,000 daltons. The native enzyme was shown to contain 4 oligosaccharide chains each averaging about 14 mannose residues. On treatment of mung bean nuclease I with the endoglycosidase, the molecular mass decreased from 39,000 to 31,000 daltons. The peptides produced on reduction of this enzyme with thiol were 18,700 and 12,500 daltons, indicating that carbohydrate had been present on both. Penicillium nuclease P_1 was decreased in size from 40,000 to 30,000 daltons by the endoglycosidase. Although most of the carbohydrate was removed from each of the native enzymes by the endoglycosidase, denaturation of the glycoproteins was necessary to effect complete removal. Enzyme activity was not affected by carbohydrate depletion of these glycoproteins, a result consistent with similar studies on other oligosaccharide-containing enzymes.

INTRODUCTION

The association of carbohydrate with protein often impedes the physical and structural characterization of these molecules. Although progress has been made in analyzing the oligosaccharide portion of many glycoproteins, this has been at the expense of the protein moiety, which must be extensively hydrolyzed by proteolytic enzymes. Alternatively, the protein can be isolated intact by prolonged treatment with the appropriate glycosidases. In each case one component must be destroyed in order to obtain the other. With the discovery of endo-β-N-acetylglucosaminidase H (1), however, this problem may at times be eliminated since oligosaccharides can be removed from intact proteins without damage to either. While other endoglycosidases (2-4) can affect similar cleavages, none appear to be as versatile as the enzyme from Streptomyces plicatus. Thus as shown recently (5), this enzyme enabled us to remove all of the carbohydrate from the external invertase of yeast, revealing for the first time that this well-known enzyme is com-

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posed of two identical subunits, each associated with 9 oligosaccharide chains. In this communication we will show the added utility of the S. plicatus endoglycosidase in further characterizing the number and composition of the oligosaccharide chains associated with yeast carboxypeptidase Y. In addition, the endoglycosidase has been used to demonstrate molecular weight changes that occur on removal of oligosaccharide from mung bean nuclease I, and penicillium nuclease P_1.

MATERIALS AND METHODS

Endo-β-N-acetylglucosaminidase H was purified to homogeneity from cultural filtrates of S. plicatus, previously referred to as S. griseus (1) but more recently identified as the indicated organism (4). For carbohydrate removal, reaction mixtures contained 0.05 to 0.08 M sodium citrate, pH 5.5, and any salts already present in the glycoprotein sample. In some instances, glycoproteins were partially unfolded by heating for 3 min at 100°C in 0.1 M mercaptoethanol-0.2% SDS[1] before adding endoglycosidase. A ratio of 0.1 to 0.2 unit (5 to 10 μg) of endoglycosidase was used for each mg of glycoprotein digested at 37°C. Carboxypeptidase Y was chromatographed at room temperature using a Sepharose 6B column (0.9 x 135 cm) equilibrated in and eluted with 20 mM Tris·HCl-100 mM NaCl, pH 7.1. When carboxypeptidase Y was denatured in SDS-mercaptoethanol, the column and elution buffer was supplemented with 0.05% SDS.

Carboxypeptidase Y was purified by a modification of described procedures (6, 7) by Dr. Thomas H. Plummer, Jr., of the Division of Laboratories and Research. Amino acid analyses of the native and endoglycosidase-treated carboxypeptidase Y were found to be identical with those previously reported (6, 7), and the residue recoveries of both the native and treated enzymes provided a specific absorbance value of $A^{1\%}_{280\ nm}$=20.12. A value of 15.0 was obtained earlier for carboxypeptidase Y which had been dried to constant weight but not corrected for its carbohydrate content (6).

Mung bean nuclease I and penicillium nuclease P_1 were purified in the Laboratory of Enzymology, Roswell Park Memorial Institute, Buffalo. Comparison of the substrate specificities of the native and carbohydrate-depleted nuclease I and P_1 was carried out at Roswell Park.

Amino acid composition was determined as described (5) on a Joelco 5AH analyzer equipped with the Durram DC1A single-column system. Glucosamine analyses on acid hydrolyzed samples (5) were performed on an amino acid analyzer using Beckman PA-35 resin and glucosaminitol as an internal standard (8). Recoveries from all analyses were 95 to 98%. By use of a carbohydrate analyzer (9) mannose was found to be the only neutral sugar associated with carboxypeptidase Y. Thus the phenolsulfuric acid assay (10) was used for hexose analysis with mannose as a standard.

Gel electrophoresis in the presence of SDS was performed as described by Weber and Osborn (11). Molecular weights were estimated by comparing migrations with polymerized lysozyme markers purchased from BDH Chemicals Ltd. Gels were

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[1]Abbreviations used are: SDS, sodium dodecyl sulfate; GlcN, glucosamine; Man, mannose; CPase, carboxypeptidase.

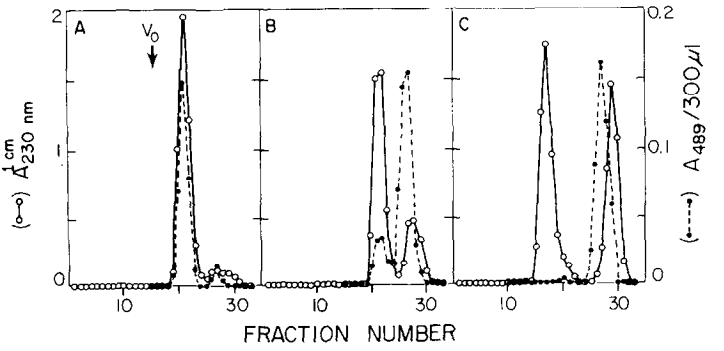

Figure 1. Chromatography on Sepharose 6B of carboxypeptidase Y before and after digestion with S. plicatus endoglycosidase. Elution profiles of: (A) 2 mg of untreated carboxypeptidase Y; (B) 2 mg of carboxypeptidase Y digested with 0.3 unit (15 µg) of endoglycosidase for 6 h at 37°C; and (C) 2 mg of carboxypeptidase Y, heated in mercaptoethanol-SDS prior to digestion with 0.3 unit of endoglycosidase for 90 min at 37°C. Three-ml fractions were collected and their absorbance was measured at 230 nm (o). The first (o) peak is due to protein, the second to salt and mercaptoethanol. Hexose (•) was measured with the phenolsulfuric acid assay.

stained with Coomassie brilliant blue and destained by diffusion into 5% methanol-7.5% acetic acid.

RESULTS AND DISCUSSION

Carboxypeptidase Y - The enzyme purified from baker's yeast is a glycoprotein of 61,000 daltons (6, 7) containing 13 to 15% neutral hexose and 2.6 (7) to 5.2% (6) amino sugar. From its compositional analysis, the protein moiety of carboxypeptidase Y is about 50,000 daltons (6, 7). Since SDS-acrylamide gels often provide inaccurate molecular weights for intact glycoproteins, it was desirable to determine how closely the molecular weight of carbohydrate-free carboxypeptidase, obtained by SDS-acrylamide gel analysis, compares with that from the amino acid composition of the native enzyme. In an effort to obtain carbohydrate-free carboxypeptidase Y, the protease was treated with endo-β-N-acetylglucosaminidase H and the digest was resolved into distinct protein (A_{230}; tubes 20-24) and carbohydrate fractions (A_{489}; tubes 25-29) by Sepharose 6B chromatography (Fig. 1B). Comparison with undigested carboxypeptidase (Fig. 1A) reveals that 75% of the hexose was removed by the endoglycosidase.

Table I. Carbohydrate Content of Carboxypeptidase Y Fractions Before and After Endoglycosidase Treatment

Enzyme Preparation	CPase Y Molar Ratio[a]		
	GlcN	Man	Mannosyl Chains
Native	7.90	55	4
Endoglycosidase-treated	4.95	15	1
Denatured and endoglycosidase-treated	3.72	0	0

[a] Based on a peptide molecular weight of 51,000.

To overcome possible steric effects which could limit the complete endoglycolytic removal of oligosaccharide, carboxypeptidase Y was unfolded in mercaptoethanol-SDS as described above and digested for 90 min at 37°C with the S. plicatus enzyme. As shown in Fig. 1C, the protein(tubes 15-20)- and hexose(tubes 25-29)-containing fractions now separated completely on Sepharose 6B chromatography Native (Fig. 1A) and carbohydrate-free (Fig. 1C) carboxypeptidases recovered from the respective columns, when subjected to SDS-acrylamide gel analysis (Fig. 2), migrated with apparent molecular weights of 61,500 (gel A) and 51,000 (gel B) respectively. The former value is in agreement with results obtained earlier by gel electrophoresis while the latter value confirms that determined from the glycoprotein composition (6, 7).

The carbohydrate content of carboxypeptidase protein fractions from the column eluates in Fig. 1 are summarized in Table I. Assuming two N-acetlyglucosamine residues per oligosaccharide chain, both in the "core" region, carboxypeptidase Y should contain 4 carbohydrate chains, each with an average of 14 mannose residues (Table I). Since endo-β-N-acetylglucosaminidase H hydrolyzes the di-N-acetylchitobiosyl core of oligosaccharide chains, the release from denatured carboxypeptidase Y of 4 of the 8 glucosamine and all 55 mannose residues was obtained as expected (Table I). Complete removal of the 4 oligosaccharide chains should

Table II. C-Terminal Amino Acid Removal from S-Carboxymethyl Invertase by Native and Carbohydrate-Depleted Carboxypeptidase[a]

Amino Acid	Molar Ratio Released by[b]	
	Native CPase Y	Treated CPase Y
Lys	1.99	2.0
Val	1.71	1.90
Ala	1.68	1.50
Ser (Gln)	1.38	1.21
Phe	0.90	0.73
Arg	0.40	0.42
All others	Trace	Trace

[a]Reactions contained 0.5 ml of 0.1 M pyridine acetate, pH 5.5, 700 µg S-carboxymethyl invertase and 4 µg of either native or treated carboxypeptidase Y After incubation at 37°C for 30 min the released amino acids were quantitated on an amino acid analyzer as described (5).

[b]Based on a holoenzyme molecular weight of 120,000 (5).

reduce the molecular weight of the carboxypeptidase by 10,000, a result confirmed by gel B, Fig. 2. Undenatured carboxypeptidase, however, was relieved of only 3 of its 4 chains (Table I), similar to studies with other native glycoproteins (5, 9).

Alkaline sodium borohydride reduction of the released oligosaccharides (Fig. 1C) indicated that all of the glucosamines are on the reducing end of these chains and are, therefore, derived from the core region. The Man/GlcN ratio of the oligosaccharide fraction in Fig. 1C was determined by analysis to be 13.5/1. From 39 nmoles of carboxypeptidase Y, 144 nmoles of this oligosaccharide was recovered, verifying the presence of 4 oligosaccharide chains per enzyme molecule as determined by analysis of the carboxypeptidase protein fractions (Table I).

To determine if removal of oligosaccharide affected carboxypeptidase Y activity, native enzyme (Fig. 1A) and enzyme depleted of 75% of its carbohydrate

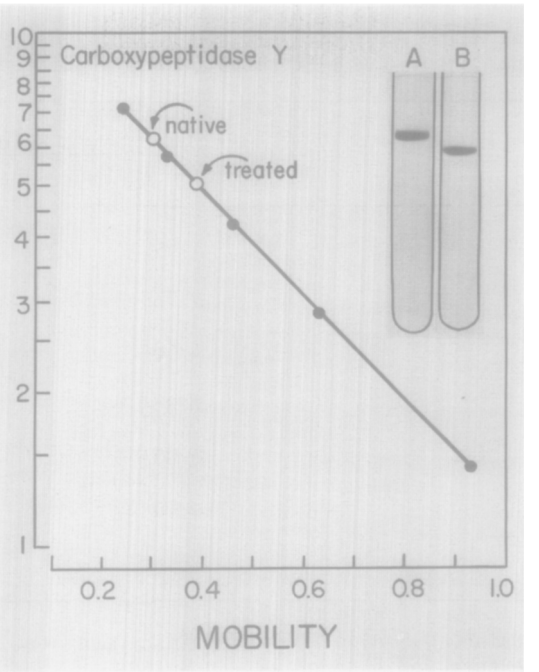

Figure 2. SDS-Acrylamide gel analysis of native (gel A) and carbohydrate-free (gel B) carboxypeptidase Y. Five µg of the protein fractions from Fig. 1A and 1C were subjected to electrophoresis on 7.5% acrylamide gels in the presence of 0.1% SDS. Molecular weight markers (●).

(Fig. 1B) were compared in their ability to remove C-terminal amino acids from S-carboxymethylated yeast invertase (5). Table II reveals no apparent difference in the activity of modified or unmodified carboxypeptidase Y by this assay procedure.

While it could be argued that the decrease in the size of carboxypeptidase Y observed on gels is due to residual proteases in the endoglycosidase preparation, none has been detected. This is supported by the observations that the amino acid composition of the native and treated enzymes are the same (see Methods), that the observed (Fig. 2) and calculated molecular weights of the carbohydrate-free proteins (6, 7) are coincident, and that enzyme activity is fully retained by carboxypeptidase depleted of most of the carbohydrate (Table II).

Mung Bean Nuclease I - Even where minute quantities of material are available, S. plicatus endoglycosidase can provide valuable information about the composition

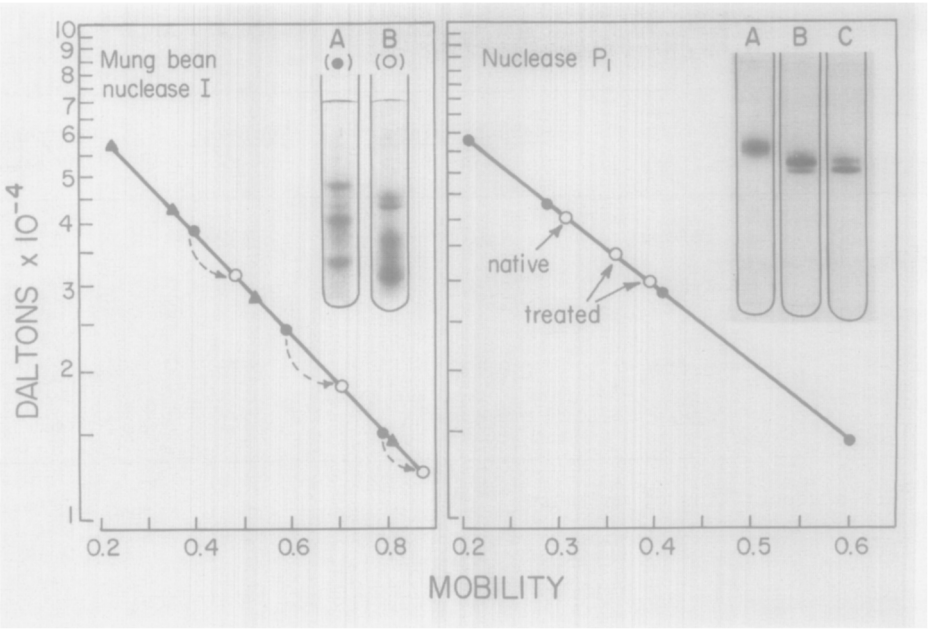

Figure 3. *Left* - SDS-Acrylamide gel analysis of 15 µg of mung bean nuclease I incubated in the presence and absence of 4 munits (0.2 µg) of S. plicatus endoglycosidase at 37°C. After 12 h the 30 µl reactions were supplemented with SDS and mercaptoethanol to 1% and heated at 100°C for 3 min. Samples were electrophoresed on 9% acrylamide gels containing 0.1% SDS. *Right* - SDS-Acrylamide gel electrophoresis of penicillium nuclease P_1 before and after treatment with S. plicatus endoglycosidase. Nuclease P_1 (25 µg), either native or denatured in SDS-mercaptoethanol, was digested with 6 munits (0.3 µg) of endoglycosidase for 2 h at 37°C. The 10% acrylamide-0.1% SDS gels contained (A) 6 µg of untreated nuclease, (B) 10 µg of endoglycosidase-treated nuclease, and (C) 10 µg of denatured, endoglycosidase-treated nuclease. Molecular weight markers; *left* (▲), *right* (●).

of a glycoprotein. The sample of mung bean nuclease I sent to us was prepared by the method of Ardelt and Laskowski (12) and was electrophoretically identical with the enzyme prepared by a more simplified method (13) in that 70% of the enzyme molecules are split in a single region of this glycoprotein. The two resulting peptides of 25,000 and 15,000 daltons remain associated in an active form through disulfide bridges. Because of the limited supply of this enzyme, an accurate assessment of the molecular weight of the protein moiety of this glycoprotein has not been possible. This information could be obtained, however, by SDS-acrylamide gel electrophoresis, after removal of carbohydrate from the nuclease with the endoglycosidase. In the case of untreated nuclease (Fig. 3-*left*, gel A), three

Table III. Activity of Mung Bean and P_1 Nucleases Before and After Endoglycosidase Treatment

Enzyme		ω-Phosphatase / A_{280}	Nuclease / A_{280}	Phosphatase / Nuclease
Mung Bean Nuclease[a]	Before	4,918	2,732	1.80
	After	3,891	2,224	1.75
P_1 Nuclease[b]	Before	2,132	428	5.0
	After	1,906	311	6.1

[a] For a description of units see (13).

[b] For a description of units see (14, 15).

major bands were present, migrating at 39,000, 24,000, and 15,000 daltons as previously indicated (13). The band at 39,000 daltons is due to enzyme consisting of single intact chain. After endoglycosidase treatment, each band was decreased by about 20% to 31,000, 18,700, and 12,500 daltons, respectively. Diffuseness in the stained bands was due to the presence of citrate buffer in the samples. The proportional decrease in weight for all species indicates that the oligosaccharide chains of mung bean nuclease are present on both peptide chains, in confirmation of results obtained by staining for carbohydrate on the gels (13).

Since insufficient nuclease I was available in these studies for residual carbohydrate analysis, the endoglycosidase-treated enzyme may not be completely free of oligosaccharide. However, through the use of the endoglycosidase, an upper limit of 31,000 daltons was established for the peptide portion of this glycoprotein. Studies comparing the ratios of nuclease to phosphatase activity for both the native and carbohydrate-depleted nuclease I indicate they have similar enzymatic properties (Table III).

Nuclease P_1 - Nuclease P_1 from Penicillium citrinum has recently been shown to be a glycoprotein with an average molecular weight of 44,000 (14, 15). In order

to obtain an estimate of its carbohydrate free molecular weight, the enzyme was treated with endoglycosidase and subjected to SDS-acrylamide gel electrophoresis (Fig. 3-*right*). Native enzyme (gel A) migrated with an apparent molecular weight of 40,000. After a 2-h treatment with endoglycosidase, nuclease P_1 displayed two bands, a major one at 34,000 and a minor one at 30,000 daltons (gel B).

Yeast invertase also yielded two bands on SDS-acrylamide gels after endoglycosidase treatment of the native enzyme, a result due to the heterogeneous distribution of residual oligosaccharide on the protein (5). However, when denatured invertase was treated with endoglycosidase, oligosaccharide was completely removed and the protein migrated as a single sharp band. To determine if the heavier nuclease P_1 band (34,000 daltons) was a migration anomaly due to residual carbohydrate, enzyme was heated in SDS-mercaptoethanol as described above and then treated with endoglycosidase. As shown in Fig. 3-*right* (gel C), most of the protein now migrated as the 30,000-daltons species. These results indicate that nuclease P_1, like carboxypeptidase Y and invertase (5), contains sterically restricted oligosaccharide cores, which become accessible to the endoglycosidase on protein denaturation. Since no bands smaller than 30,000 daltons appear after denaturation, this band may represent the carbohydrate-free protein. Substrate specificity studies revealed no significant differences between the native and carbohydrate-depleted enzyme with respect to the ratio of nuclease to phosphatase activities (Table III). Thus as in the case of carboxypeptidase Y, mung bean nuclease I, and also invertase (9, 16), the carbohydrate associated with nuclease P_1 is dispensable as far as enzyme activity is concerned.

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