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Structural and Chemical Characterization of a Homogeneous Peptide *N*-Glycosidase from Almond†

Eulazio M. Taga,‡ Abdul Waheed, and Robert L. Van Etten*

ABSTRACT: A peptide *N*-glycosidase that catalyzes the hydrolysis of N-linked oligosaccharide chains from glycopeptides and glycoproteins has been purified to homogeneity from almond emulsin and from almond meal. Purification from almond emulsin using ion-exchange chromatography, gel filtration chromatography, and preparative polyacrylamide gel electrophoresis gave an enzyme which was purified more than 700-fold and with a yield of 63%. An alternative procedure, more suitable for efficient large scale purification, used ion-exchange, affinity, and gel filtration chromatography. When purification began with almond emulsin, the enzyme was purified 1200-fold with a 37% yield, while when purification began with almond powder, the enzyme was purified 9000-fold with a yield of 45%. The homogeneous enzyme is stable at 4 °C for several months in 10 mM sodium acetate, pH 5.0, buffer. The peptide *N*-glycosidase is itself shown to be a glycoprotein consisting of a single polypeptide chain with a molecular weight of 66 800 on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Circular dichroism spectra of the native molecule indicate the presence of a high (approximately 80%) α-helix content. The amino acid and carbohydrate contents of the enzyme are presented. When a convenient new assay with a turkey ovomucoid glycopeptide as a substrate is used, the enzyme preparation exhibits a broad pH optimum centered between pH 4 and pH 6. The enzyme is readily inactivated by SDS and guanidine hydrochloride, but it is stable in the presence of moderate concentrations of several other protein denaturants. Several divalent metal ions, e.g., Mg^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}, increase the enzyme activity by as much as 50%, but 10 mM ethylenediaminetetraacetic acid does not inhibit the enzyme activity. Although the enzyme cleaves the carbohydrate from a variety of glycopeptides used in this study (albeit at different rates), it does not hydrolyze the carbohydrate from most of the corresponding partially denatured glycoproteins under otherwise comparable conditions. However, the carbohydrates from denatured ovomucoid and ribonuclease B were completely removed. Following purification by affinity chromatography, the homogeneous endoglycosidase exhibits a negligible β-hexosaminidase activity, measured by using a synthetic substrate for assay.

Numerous endoglycosidases have been purified from bacterial cultures and have been found to be of great help in elucidating the structures of the oligosaccharides of glycoproteins (Tarentino et al., 1974; Tarentino & Maley, 1974; Koide & Muramatsu, 1974; Elder & Alexander, 1982). A related enzyme activity has also been reported in mammalian tissues (Tachibana et al., 1982). Endoglycosidases such as endo H, endo D, and endo F cleave high mannose, complex glycans, and both types of glycans from the native or partially denatured glycoproteins, respectively (Tarentino et al., 1974; Tarentino & Maley, 1974). However, they do not completely remove the carbohydrate residues attached to the peptide or protein chain.

Recently, a peptide *N*-glycosidase from almond emulsin has been partially purified and characterized (Takahashi, 1977; Takahashi & Nishibe, 1978; Takahashi & Nishibe, 1981; Plummer & Tarentino, 1981; Tarentino & Plummer, 1982). Almond endoglycosidase has been shown to completely remove oligosaccharides from several glycoproteins and glycopeptides (Takahashi & Nishibe, 1981). It appears that the removal of oligosaccharides from native glycoproteins is typically much slower than their removal from derived glycopeptides, at least in the cases of ovalbumin, bromelain, and desialotransferrin. By use of an enzyme immobilized on Sepharose, Takahashi et al. (1982) were able to completely deglycosylate the native enzyme takaamylase A. In contrast, Tarentino & Plummer (1982) have shown that the release of oligosaccharides from native glycoproteins is very difficult, and they were able to remove oligosaccharides from denatured glycoproteins only in the presence of chaotropic salts and disulfide bond reducing agents. To date, the properties of a homogeneous peptide *N*-glycosidase have not been reported.

Considering the contradictory facts in the literature and the great importance of having such an enzyme available in a pure state for use in studies of the role of oligosaccharides in glycoprotein structure and function, we have purified one of the endoglycopeptidases to homogeneity and have carefully studied some of its physical and structural properties as well as some of the conditions necessary for its action on glycoprotein and glycopeptide substrates. As will be evident from our study, the enzyme is itself a glycoprotein, and this important property was used to develop an affinity chromatography procedure for convenient purification of the enzyme. In addition, we also utilized a rapid and convenient assay procedure which will also

† From the Department of Chemistry, Purdue University, West Lafayette, Indiana 47907. *Received July 11, 1983.* This work was supported in part by DHHS National Institutes of Health Grant GM 27003 from the National Institute of General Medical Sciences.
‡ Permanent address: University of São Paulo and Conselho Nacional de Pesquisas (CNPq), Bauru, Brazil.

0006-2960/84/0423-0815$01.50/0

be expected to facilitate future studies.

Materials and Methods

Reagents. β-Glucosidase type II, defatted almond meal, trypsin inhibitor from turkey egg white type II-T, RNase B from beef pancreas, (SDS) sodium dodecyl sulfate molecular weight markers, Sephadex G-25, Sephacryl S-200, and DEAE-Sepharose were purchased from Sigma Chemical Co. and *S. aureus* protease and endoglycosidase H from Miles Laboratories, Inc.; acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulfate, and precast slab gels for isoelectric focusing were obtained from LKB Instruments, Inc., and CM-Bio-Gel A was from Bio-Rad Laboratories, Inc. Glycopeptide GP158 from rat submandibular gland was a gift from Prof. Don Carlson (Mehansho & Carlson, 1983). All other chemicals were of reagent grade.

Purification of a Turkey Ovomucoid Glycopeptide. Turkey ovomucoid (10 g) was dissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris) and 0.01 M $CaCl_2$, pH 8.2, to a final concentration of 2 or 4%, and *Staphylococcal aureus* protease (0.1%) was added. The solution was incubated for 48 h with stirring at room temperature. The progress of hydrolysis was checked by high-pressure liquid chromatography (HPLC), and the reaction was stopped by addition of ethylenediaminetetraacetic acid (EDTA) at a final concentration of 0.02 M. The sugar-containing peptide from the first domain was purified by gel filtration on Sephadex G-50 and DEAE-Sepharose chromatography. After the ion-exchange chromatography the sample was lyophilized and desalted on Sephadex G-25. The desalted glycopeptide sample was lyophilized and stored at 4 °C. The glycopeptide yield was 200 mg/14 g of turkey ovomucoid. The glycopeptide contains 25% neutral sugar which was resistant to endoglycosidase H, indicating a complex type of glycan. The amino acid sequence of the peptide is H_2N-Tyr-Gly-Thr-Asn(CHO)-Ile-Ser-Lys-Gly-COOH (W. M. Westler and W. C. Bogard, Jr., unpublished results).

Protein Determination. The protein concentration was determined by the method of Lowry et al. (1951) with crystalline bovine serum albumin as a standard. The protein profile of chromatographic steps was monitored by measuring the absorbance at 280 nm using a Gilford 2000 spectrophotometer.

Analytical Polyacrylamide Gel Electrophoresis. Electrophoresis of the native enzyme in polyacrylamide gel was performed in 7.5% gels at pH 8.9 (Davis, 1964) and at pH 4.5 (Reisfeld et al., 1962).

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Sodium dodecyl sulfate electrophoresis was conducted according to the method of Weber & Osborn (1969) and SDS slab gel electrophoresis was done according to Laemmli (1970).

β-N-Acetylglucosaminidase (β-Hexosaminidase) Activity. The β-hexosaminidase activity was determined at 37 °C by using 0.5 mL of 3 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 100 mM acetate buffer, pH 4.5, containing 1 mg/mL bovine serum albumin. After incubation for 5–30 min the reaction was stopped by addition of 1.0 mL of 1.0 N NaOH. The absorbance was read at 400 nm, and 1 unit of activity is defined as 1 μmol of *p*-nitrophenol liberated/min. The molar extinction coefficient of *p*-nitrophenoxide used in calculations was 1.8×10^4 M^{-1} cm^{-1}. Specific activity is defined as the number of enzyme units per milligram of protein. Competitive inhibition by *N*-acetylgalactosamine was measured with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as a substrate.

Peptide N-Glycosidase Activity. The enzyme activity was determined at 37 °C in 0.1 mL of assay mixture containing 125 μg of ovomucoid glycopeptide in 20 mM acetate buffer, pH 5.0, containing phenylmethanesulfonyl fluoride (1 mM), pepstatin (1 μg/mL), and iodoacetate (1 mM) in small polyethylene centrifuge tubes. After incubation for 2 h at 37 °C the reaction was stopped by addition of 1.0 mL of ethanol at −20 °C. The tubes were left for 30 min at −20 °C and then centrifuged in a small Eppendorf centrifuge for 2 min. The supernatant was carefully collected, and the liberated sugar was determined by the phenol–sulfuric acid method using a 0.5-mL portion of the supernatant. The absorbance at 480 nm was read against a blank containing 0.5 mL of alcohol. One enzyme unit is expressed as 1 nmol of neutral sugar (mannose standard) liberated/min. Specific activity is defined as the number of enzyme units per milligram of protein.

Two other methods were also utilized. The hydrolysis of ovomucoid glycopeptide was followed by measuring the amount of ammonia released during hydrolysis of the terminal peptide N-glycosidic linkage (Takahashi & Nishibe, 1981). The peptide *N*-glycosidase activity was also monitored by high-pressure liquid chromatography using a TSKg 3000 SW column. After sample injection (5–10 μL) the column was developed by using 0.2 M potassium phosphate buffer, pH 6.5. The effluent was monitored at 206 nm. The flow rate was adjusted to 1.0 mL/min, the absorbance at 206 nm to 0.1 optical density full scale, and the recorder speed to 0.5 cm/min.

Removal of Carbohydrate from Glycoproteins. The release of oligosaccharide from intact glycoproteins due to action of the enzyme was monitored by SDS slab gel electrophoresis. The ovomucoid was first denatured in either 9 M urea, 6 M guanidine hydrochloride, 3 M potassium thiocyanate, 3 M sodium perchlorate, 4 M lithium chloride, 1% SDS, 2% Triton X-100, 2% Tween-80, 2% dodecyltrimethylammonium bromide, or 2% Zwittergent in 50 mM sodium acetate, pH 5.0, with 4 mM phenylmethanesulfonyl fluoride (PMSF) containing 0.5 M β-mercaptoethanol. In order to ensure equilibration, the protein solution was kept at room temperature with the denaturants for at least 24 h. Other glycoproteins such as ovalbumin, human prostatic acid phosphatase, orosomucoid, and ribonuclease B were predenatured in 9 M urea in 50 mM sodium acetate, pH 5.0, plus 4 mM PMSF containing 0.5 M β-mercaptoethanol. An aliquot of predenatured glycoprotein (80 μg of protein) was treated with pure enzyme (8 μg) in 25 mM sodium acetate, pH 5.0, plus 4 mM PMSF buffer at 37 °C for 96 h. The final concentration of denaturants in the assay mixture was the one which was found to be noninhibitory to the enzyme (see Results). Approximately 30 μL of sample was denatured in an equal volume of solubilizer at 95 °C for 10 min and used for SDS, slab gel electrophoresis. Two parallel gels were run; one was stained for protein with Coomassie blue and the other for carbohydrate with the Schiff–periodate reagent. Inhibition of the enzyme by *N*-acetylgalactosamine was also examined by slab gel electrophoresis using ribonuclease B as a substrate. The SDS slab gel was scanned by using a densitometer (E-C Apparatus Corp.), and the percent activity corresponding to each peak was obtained quantitatively by using a Du Pont curve resolver.

Amino Acid and Carbohydrate Analyses. Analyses were performed on a Durrum D-500 amino acid analyzer. Samples (100 μg) were subjected to acid hydrolysis by using constant boiling 6 N HCl at 110 °C for 24, 48, and 72 h. The number of residues per molecule was determined by using the integer-fit method of Hoy et al. (1974). The values for serine, threonine, and tyrosine were corrected for decomposition by extrapolation to zero time of the values obtained at 24, 48, and 72 h. The carbohydrate composition of the homogeneous

glycopeptidase was determined by gas chromatography of the alditol acetate derivatives according to a published procedure (Weber & Carlson, 1982). Neutral sugar was determined by a modified phenol–sulfuric acid method (McKelvy & Lee, 1969) with mannose as a standard.

Isoelectric Focusing. The isoelectric focusing was done by using precast gels provided by LKB Instruments Co. and following the directions given by the manufacturer.

Circular Dichroism Measurements. Circular dichroism experiments were performed at room temperature on a Cary 61 spectropolarimeter using a 1-cm light path cell. Mean residue ellipticities, $[\theta]$ (in deg cm^2 dmol^{-1}) were calculated from the measured ellipticities, θ, by the equation $[\theta]_\lambda = [(\theta/10)[M_0/(lc)]]$ where θ is measured ellipticities in degrees, l is the optical path length in centimeters, c is the protein concentration in grams per milliliter, and M_0 is the mean residue weight (molecular weight divided by the number of amino acid residues), which is 113.

Fluorescence Measurements. Fluorescence spectra were recorded on a Perkin-Elmer MPF-44A fluorescence spectrometer. Emission and excitation slit widths were kept constant at 6 nm. Protein samples were excited at 295 nm, and uncorrected spectra were plotted.

Effect of Protein Denaturants on Peptide N-Glycosidase Activity. Protein denaturants at different concentrations were incubated with 10 μg of the enzyme and 100 μg of ovomucoid glycopeptide. Hydrolysis was carried out for 2 h at 37 °C, and the reaction was stopped by addition of 1.0 mL of ethanol. Total carbohydrate in the supernatant was estimated by using the phenol–sulfuric acid method. Protein denaturants used were guanidine hydrochloride (0.9–14%), SDS (0.01–1%), urea (0.6–12%), lithium chloride (0.4–8.5%), β-mercaptoethanol (0.08–3.9%), Triton X-100 (0.1–5%), Tween 80 (0.1–2%), Nonidet P-40 (0.1–5%), dodecyltrimethylammonium bromide (0.1–2%), or Zwittergent (0.1–1%).

Enzyme Purification. One early purification of the enzyme was carried out by starting with 3 g of crude almond emulsin (Sigma Chemical Co.; type II β-glucosidase) and utilizing a sequence of steps including extraction, Sephadex G-25, DEAE-Sepharose, Sephacryl S-200, and SP-Sephadex column chromatography, and finally preparative electrophoresis. By use of these steps, a homogeneous peptide *N*-glycosidase having a specific activity of 6.0×10^3 units/mg was obtained with a recovery of 63%. This preparation was used for most of the experiments described here with the exception of the fluorescence and circular dichroism experiments. Subsequent studies revealed that this enzyme is a glycoprotein, and this finding in turn permitted the development of more efficient purification protocols such as the following protocol beginning with alternative starting materials.

(*1*) *Aqueous Extract.* Either defatted almond meal (200 g in 1 L of 5 mM Tris-HCl, pH 8.8) or almond emulsin (2 g in 20 mL of 5 mM Tris-HCl, pH 8.8) was suspended in buffer and extracted by stirring at 4 °C for 24 h. The supernatant was recovered by centrifugation at 9000 rpm for 30 min in a Beckman JA-10 rotor. This and all subsequent buffers contained 4 mM PMSF.

(*2*) *DEAE-Sepharose Chromatography.* A column of DEAE-Sepharose (5 × 4.5 cm) was equilibrated with 5 mM Tris-HCl, pH 8.8, and the enzyme preparation from step 1 was applied. The column was washed with two bed volumes of the initial buffer, and then bound protein was eluted with 150 mM sodium chloride/5 mM Tris-HCl, pH 8.8. Most of the enzyme activity was recovered in this fraction (Figure 1). (The column was finally washed with 500 mM sodium chloride, but this fraction did not show any activity.) The enzyme-containing fraction was treated at 4 °C with ammonium sulfate (500 g/L of solution), and the resulting suspension was allowed to stand for at least 6 h. The protein precipitate obtained after centrifugation was dissolved in 5 mM sodium phosphate, pH 7.0, buffer.

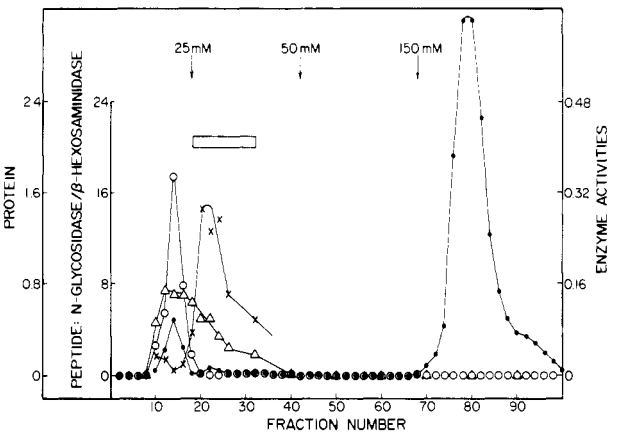

FIGURE 1: DEAE-Sepharose chromatography of peptide *N*-glycosidase. The enzyme preparation from step 1 was applied to a DEAE-Sepharose column (5 × 4.5 cm). Fractions of 8.0 mL were collected and monitored for peptide *N*-glycosidase activity at 480 nm (△), β-hexosaminidase activity at 405 nm (○), and protein concentration at 280 nm (●). The ratio of the peptide *N*-glycosidase to β-hexosaminidase activities is also plotted (×). The arrows indicate different millimolar concentrations of NaCl. Fractions under the solid bar were pooled for further purification.

(*3*) *Concanavalin A–Sepharose Chromatography.* The preparation from step 2 was loaded on a column (4.5 × 5 cm) which was equilibrated with 5 mM sodium phosphate, pH 7.0. The column was washed with five bed volumes of initial buffer followed by 1 M sodium chloride/5 mM sodium phosphate, pH 7.0, at room temperature. Washing of the column was continued until the absorbance at 280 nm fell below 0.01. The bound glycoproteins were eluted with three bed volumes of 1 M sodium chloride and 10% mannose in 5 mM sodium phosphate, pH 7.0. The protein obtained was precipitated with ammonium sulfate (500 g/L of solution). The protein precipitate was recovered, dialyzed against 20 mM Tris-HCl, pH 7.5, and concentrated by using an Amicon ultrafiltration assembly using a PM-10 membrane.

(*4*) *Sephacryl S-200 Chromatography.* A 9.0-mL protein sample was applied to a 2.5 × 150 cm column equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The fractions were monitored for the enzyme activity, and protein was concentrated by using Amicon ultrafiltration. The enzyme sample was dialyzed against 10 mM sodium acetate, pH 5.0.

(*5*) *CM-Bio-Gel A Chromatography.* A small column (0.5 × 1.5 cm) in a disposable Pasteur pipet was packed with CM-Bio-Gel A resin and equilibrated with 10 mM sodium acetate, pH 5.0. The preparation from step 5 was applied, and the column was washed with 2 mL of initial buffer. The protein was eluted with stepwise elution using 2 mL each of 50, 100, and 200 mM sodium chloride dissolved in the initial buffer. Fractions were monitored for protein and the enzyme activities. Contaminating proteins were eluted in the breakthrough and in the first wash, but these fractions did not show any activity. The enzyme eluted under all three salt concentrations showed both hexosaminidase and endoglycosidase activities. The specific activity was constant for all fractions. These fractions were pooled and concentrated.

Results

The purification procedure is outlined in Table I. The pure

Table I: Purification of a Peptide *N*-Glycosidase from Almond Meal[a]

step	volume (mL)	protein (mg)	endo-β-*N*-acetylglucosaminidase (units)	peptide *N*-glycosidase (units)	specific activity (units/mg)[b]	recovery (%)
aqueous extract	480	69 940[c]	3414	39 750	0.61	
concanavalin A–Sepharose	89	1688	62.3	37 473	22	(100)
DEAE-Sepharose	15.2	42	0.67	25 410	6.1×10^2	68
Sephacryl S-200	2.6	8.0	0.04	20 893	2.6×10^3	56
CM-Bio-Gel A	2.5	2.9	0.003	17 000	5.7×10^3	45

[a] An alternative purification procedure, starting with 2 g of commercial almond emulsin and reversing the order of the concanavalin A–Sepharose and DEAE-Sepharose steps, yielded 3.0 mg of enzyme with a specific activity of 6.2×10^3 units/mg. [b] Peptide *N*-glycosidase activity assayed by using a turkey ovomucoid substrate. [c] Starting with 200 g of fat-free almond meal.

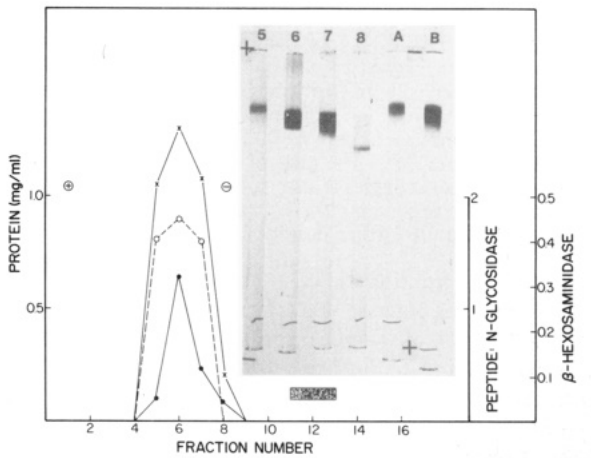

FIGURE 2: Preparative polyacrylamide gel electrophoresis of peptide *N*-glycosidase. The enzyme preparation was loaded in a 1.5 × 12 cm tube of polyacrylamide gel. After electrophoresis, the gel was frozen and cut into 4-mm slices. The protein was extracted with 4 mL of 0.02 M acetate buffer, pH 5.0. The protein and enzyme activities were determined in each fraction: protein (●), peptide *N*-glycosidase (○), and β-hexosaminidase (×). The solid bar indicates fractions with precipitated proteins. The inset shows the electrophoretic pattern at pH 4.5 of each fraction indicated by a number. Gels A (12.5 μg) and B (25 μg) show electrophoresis of the enzyme preparation at pH 8.6.

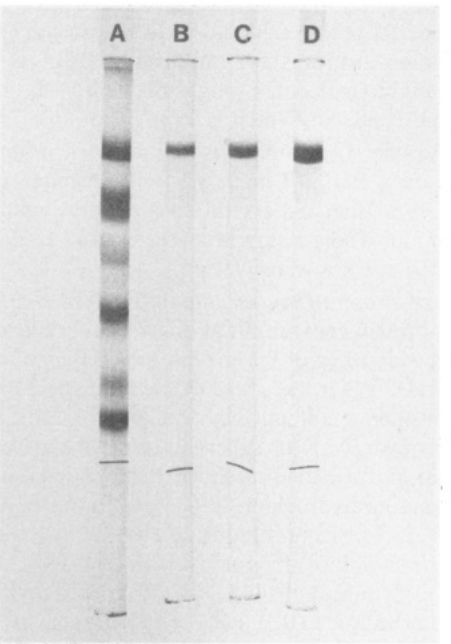

FIGURE 3: SDS–polyacrylamide gel electrophoresis of peptide *N*-glycosidase. Electrophoresis was performed with standard proteins (A) [bovine serum albumin (69 000), human prostatic acid phosphatase (54 000), ovalbumin (45 000), pepsin (34 700), trypsinogen (24 000), β-lactoglobulin (18 400), and lysozyme (14 300)] and different amounts of pure enzyme [5 (B), 10 (C), and 15 μg (D)]. The calibration curve fitted a linear equation, $\log M_r = 5.05 - R_f$ with a regression coefficient of 0.99. The relative mobility (R_f) of the enzyme was 0.225.

enzyme has a final specific activity of 6000 units/mg, and the overall yield varies from 37 to 63%. The purification factor varies from 700- to 1200-fold when almond emulsion is used as a starting material but is over 9000-fold starting from almond meal (Table I). The enzyme is stable for several months when stored at 4 °C in 10 mM acetate buffer, pH 5.0, but partial inactivation (of both activities; see below) was observed when the enzyme was stored with 50% glycerol or without glycerol at −20 °C.

Polyacrylamide gel electrophoresis of the native molecule at pH 8.6 and at pH 4.5 showed a single protein band (Figure 2). The homogeneity of the enzyme was also tested by using SDS gel electrophoresis with increasing amounts of protein (Figure 3). A single protein band was observed. The molecular weight of the protein obtained by SDS gel electrophoresis was found to be 66 800 under reducing conditions (Figure 3) and 89 000 under nonreducing conditions. The amino acid composition is shown in Table II. Using data obtained for the amino acid analysis of the purified protein, we estimate a molecular weight of 58 896 for the protein portion of the molecule. In isoelectric focusing the enzyme appears as a broad band between pH 7.0 and pH 7.7, indicating that this enzyme has some charge heterogeneity.

The dependence of activity upon enzyme protein concentration shows that the reaction is linear for enzyme concentrations less than 10 μg/mL. A time dependence study shows that the activity toward ovomucoid glycopeptide is linear with time for at least 2.5 h when the enzyme concentration is 10 μg/mL.

The pH dependence on the enzyme activity shows that the enzyme has a broad pH optimum between pH 4 and pH 6 (Figure 4) when the glycopeptide from ovomucoid is used as a substrate. The enzyme activity was reduced by 50% at pH 3.0, and there was no significant activity against this substrate at pH 7.5.

The stability of enzyme was tested toward several chemicals known to denature glycoproteins, since denaturing conditions should be useful to further expose the carbohydrates present on the glycoproteins. The enzyme is active in the presence of several relatively concentrated denaturants including Triton X-100, Tween 80, dodecyltrimethylammonium bromide, Zwittergent, Nonidet P-40, urea, β-mercaptoethanol, and lithium chloride. However, SDS readily inactivates the enzyme; at an SDS concentration of 0.01%, only 20% of the activity is retained by the enzyme. It is also partly inactivated by guanidine hydrochloride: at 1.5 M guanidine hydrochloride only 40% of the hydrolase activity remains under the assay conditions tested. Although some cations such as Mg^{2+}, Zn^{2+},

Table II: Peptide *N*-Glycosidase Amino Acid and Carbohydrate Composition

residue	no.[a]	residue[b]	no.
aspartic acid	66	*N*-acetylglucosamine	16
threonine	27	fucose	11
serine	41	mannose	21
glutamic acid	43		
proline	27		
glycine	45		
alanine	35		
cysteine	9		
valine	32		
methionine	6		
isoleucine	24		
leucine	44		
tyrosine	36		
phenylalanine	26		
histidine	17		
lysine	33		
arginine	18		
tryptophan	*c*		
glucosamine	18		
total no. of residues	520		
M_r due to amino acid residues	58896		

[a] Per molecule, based on the integer-fit method of Hoy et al. (1974). [b] Determined by gas chromatographic analysis (Weber & Carlson, 1982) of the alditol acetate derivatives starting with 1.0 mg of homogeneous enzyme. [c] Not determined.

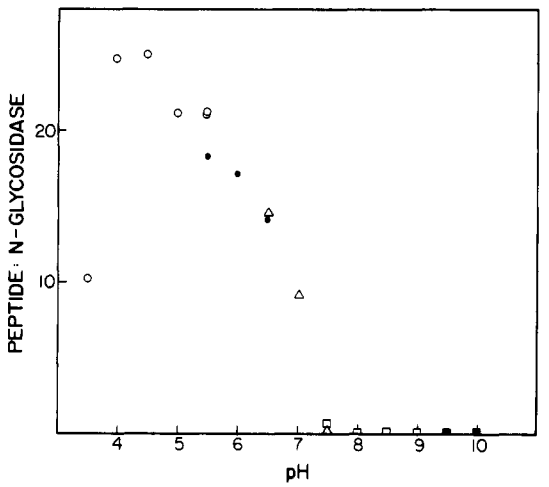

FIGURE 4: Effect of pH on the enzyme activity. The enzyme activity was measured in buffer (50 mM) of different pH values. The assay mixture consisted of 100 μg of ovomucoid glycopeptide and 10 μg of the enzyme, and the reaction was carried out for 2 h at 37 °C. The reaction was stopped by addition of 1.0 mL of ethanol, and the released sugar was determined by the phenol–sulfuric acid method. The enzyme activity was expressed as micrograms of mannose released. Different buffers used were sodium acetate (○), sodium cacodylate (●), sodium phosphate (△), Tris-HCl (□), and barbital–sodium hydroxide (■).

Co^{3+}, and Cu^{2+} increase the enzyme activity by as much as 50%, the endoglycopeptidase is not inhibited by 10 mM EDTA.

High-pressure liquid chromatographic analysis of reaction mixtures obtained during enzymatic hydrolysis of the ovomucoid glycopeptide substrate quickly demonstrates the course of the reaction (Figure 5). During reaction, the peak corresponding to intact glycopeptide (retention time 17.5 min) gradually disappears with the simultaneous appearance of lower molecular weight products (retention times 19.25 and 20.5 min). The product with a retention time of 20.5 min no longer exhibits a positive Schiff test. Related large-scale experiments with ribonuclease B also demonstrated that the

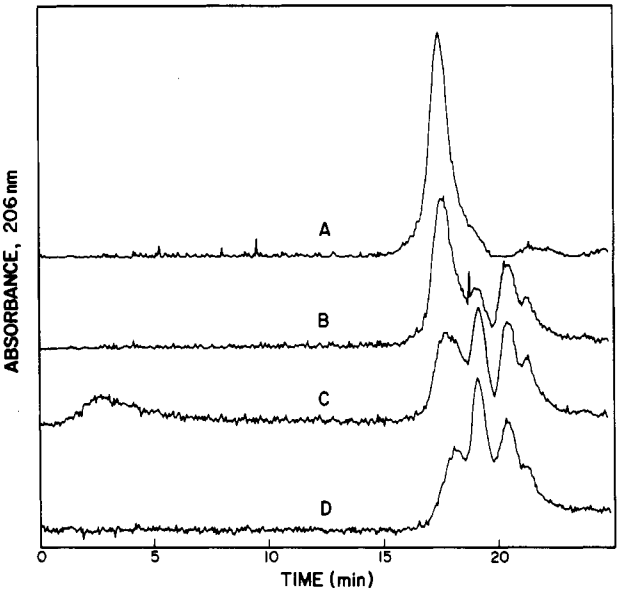

FIGURE 5: HPLC traces demonstrating the enzyme-catalyzed cleavage of turkey ovomucoid glycopeptide and the release of the peptide and carbohydrate components. Ovomucoid glycopeptide (200 μg) was treated with enzyme (5 μg) in a final volume of 0.1 mL at 37 °C. A 10-μL aliquot of the reaction mixture at different time intervals was subjected to chromatography. The chromatographic patterns are shown at zero time (A) and after 30 (B), 60 (C), and 180 min (D). Irregular subtraction of a peak due to buffer components (established by injection of buffer alone) causes the apparent band at 22 min corresponding to the internal volume of the column.

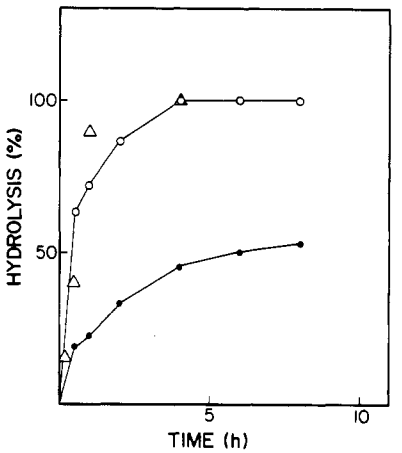

FIGURE 6: Time course for hydrolysis of ovomucoid and ovalbumin glycopeptides by the enzyme. Enzyme (10 μg) was incubated with 100 μg each of ovomucoid glycopeptide (○) and ovalbumin glycopeptide (●) in a final volume of 0.1 mL in 20 mM acetate buffer, pH 5.0. At different times the reaction was stopped by addition of 1.0 mL of ethanol, and free carbohydrate was determined by using the phenol–sulfuric acid method. The percent hydrolysis was calculated on the basis of the total amount of carbohydrate present in glycopeptides. Under identical conditions, the hydrolysis of ovomucoid glycopeptide could also be followed by measuring the release of ammonia (△).

enzyme completely removes the carbohydrate.

Figure 6 shows that the homogeneous enzyme hydrolyzes ovomucoid glycopeptide (with a complex oligosaccharide) at a velocity faster than that exhibited with ovalbumin glycopeptide (which contains a high mannose type of carbohydrate). Thus, in 30 min the turkey ovomucoid and the ovalbumin glycopeptides were cleaved to the extent of 62 and 20%, respectively. At long times only the ovomucoid glycopeptide is completely cleaved (measured by two methods), further indicating its suitability as a substrate. In other experiments, we found that glycopeptide GP158 from rat submandibular

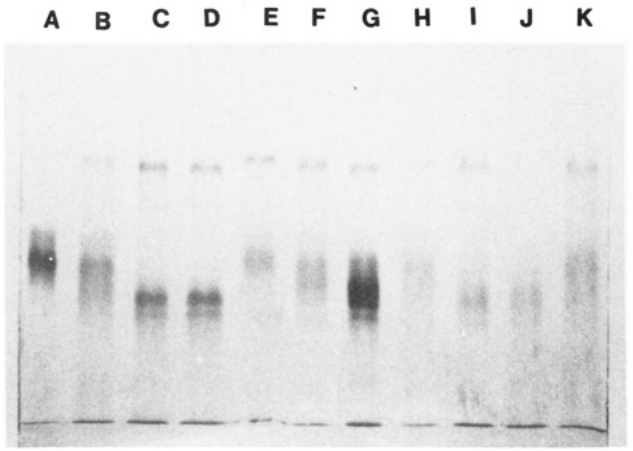

FIGURE 7: SDS–polyacrylamide gel electrophoresis patterns of ovomucoid exposed to peptide *N*-glycosidase in the presence of different denaturants. Lane A, Native protein; lane B, SDS; lane C, Triton X-100; lane D, Tween 80; lane E, DTAB; lane F, Zwittergent; lane G, urea; lane H, guanidine hydrochloride; lane I, potassium thiocyanate; lane J, sodium perchlorate; lane K, lithium chloride.

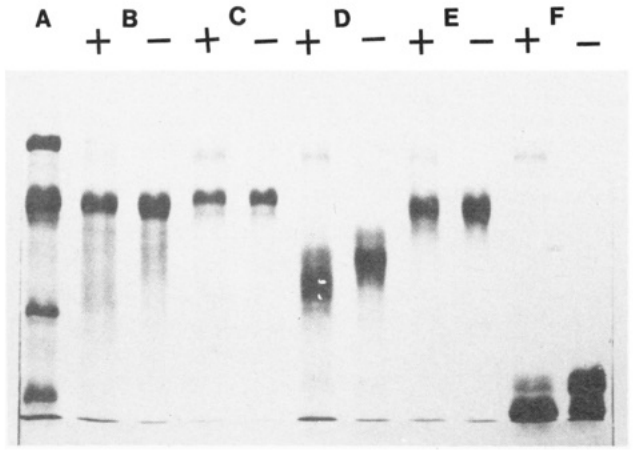

FIGURE 8: SDS–polyacrylamide gel electrophoresis of glycoproteins exposed to peptide *N*-glycosidase in the presence of urea. Lane A, standard proteins (see Figure 6); lane B, ovalbumin; lane C, human prostatic acid phosphatase; lane D, ovomucoid; lane E, orosomucoid; lane F, ribonuclease B. (+) and (−) indicate the presence and absence of the peptide *N*-glycosidase.

gland is hydrolyzed even more slowly than the ovalbumin glycopeptide (15% in 8 h).

Ovomucoid was subjected to different denaturants in the presence of 0.5 M β-mercaptoethanol and then incubated with the purified glycosidase. Experimental results obtained by SDS–PAGE are illustrated in Figure 7. The ovomucoid denatured in Triton X-100, Tween 80, urea, potassium thiocyanate, or sodium perchlorate and incubated with the glycosidase was then found to migrate as a low molecular weight protein product, consistent with cleavage of oligosaccharide from the glycoprotein. There was a partial reaction of ovomucoid which had been treated with SDS, Zwittergent, guanidine hydrochloride, or LiCl and then incubated with the glycosidase. Ovomucoid exposed to dodecyltrimethylammonium bromide was not affected. This is probably due to incomplete denaturation of ovomucoid since we established independently that the enzyme itself is not inactivated in the presence of this detergent.

On the basis of this study of the effects of various denaturants, we attempted to cleave several glycoproteins in urea solution containing β-mercaptoethanol (Figure 8). Except for ovomucoid and ribonuclease B, the other native glycoproteins that were tested were not hydrolyzed by the endo-

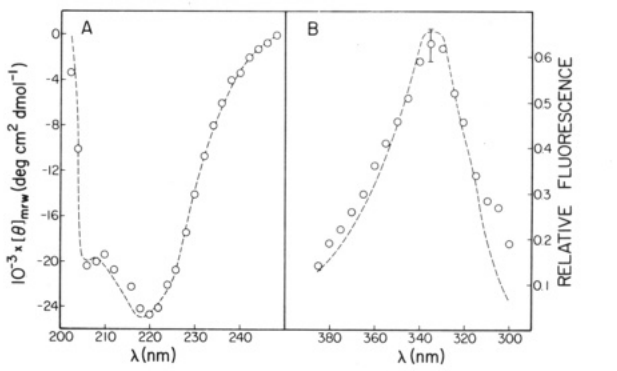

FIGURE 9: Spectral profiles of the homogeneous peptide *N*-glycosidase. (A) Circular dichroism spectra: native peptide *N*-glycosidase in 10 mM sodium acetate, pH 5.0 (---); enzyme in 2 M urea in 10 mM sodium acetate, pH 5.0 (O). (B) Fluorescence emission spectra: native peptide *N*-glycosidase in 10 mM sodium acetate, pH 5.0 (---); enzyme in 2 M urea, 1.5 M guanidine hydrochloride, or 0.2% sodium dodecyl sulfate, each in 10 mM sodium acetate, pH 5.0 (O).

glycosidase. This may be due to an inaccessibility of the carbohydrate residues in these glycoproteins.

The homogeneous enzyme was devoid of measureable α-mannosidase and β-galactosidase activity and exhibited only traces of a β-hexosaminidase type of activity (Table I). Both K_m and V_{max} for the β-hexosaminidase and the endoglycosidase activities appear to be influenced by enzyme concentration. Thus, the peptide *N*-glycosidase activity was 3-fold higher at 0.06 μg/mL protein than at 0.6 μg/mL. The elution volume of the enzyme on Sephacryl S-200 column was identical at 0.6 and 0.06 μg/mL protein concentrations, indicating that the peptide *N*-glycosidase does not change its molecular weight at the higher protein concentration. Some studies suggested that both the β-hexosaminidase and peptide *N*-glycosidase activities were present in the same protein. Thermal denaturation studies involving heating of the enzyme solution at 50 °C did not cause any change in the β-hexosaminidase and endoglycosidase activities, whereas heating at 60 and 70 °C inactivated both activities to a comparable extent. When the enzyme was incubated with known inhibitors of β-hexosaminidase such as 0.1 mM Ag^+ or 3 mM *p*-(hydroxymercuri)phenylsulfonate, both activities were completely inhibited. *N*-Acetylgalactosamine is a competitive inhibitor of both the hexosaminidase and endoglycosidase activities, although the K_i value for inhibition of the hexosaminidase activity was 5 mM, which is around 10-fold higher than the K_i determined for other hexosaminidases (Geiger & Arnon, 1978). However, the K_i value for inhibition of the endoglycosidase activity is 174 mM. Moreover, the amount of β-hexosaminidase activity relative to the peptide *N*-glycosidase activity varies widely during the purification process (cf. Figure 1) and is sharply reduced following affinity chromatography (Table I), indicating the separation of distinct proteins.

In view of the unusual stability of the enzyme molecule toward many denaturants, and its inactivation by low concentrations of sodium dodecyl sulfate (0.01%) and guanidine hydrochloride (1.5 M), the spectral properties of the enzyme were examined (Figure 9) to obtain information about the structure of the endoglycosidase. A fluorescence spectrum is shown in Figure 9B. The native endoglycosidase shows a typical emission maximum near 335 nm for proteins containing tryptophan, and this maximum was not influenced by 2 M urea, 1.5 M guanidine hydrochloride, or 0.2% sodium dodecyl sulfate. Fluorescence intensity was not significantly changed due to these denaturants since the small variation that was observed was within experimental error.

The circular dichroism spectra are shown in Figure 9A. The native molecule exhibits a minimum at 218 nm and a shoulder near 208 nm. The optical ellipticity of the endoglycosidase near 222 nm was −24 000 deg cm^2 dmol^{-1} which is consistent with an 80% helix content in the native molecule (Chen et al., 1974). There was no change in the spectrum in the presence of 2 M urea; small variations around the minima were well within experimental error.

Discussion

In recent years several specific glycosidases have proven important in the study of the structure and functions of carbohydrate of biologically important glycoproteins (Tarentino et al., 1974; Tarentino & Maley, 1974; Koide & Muramatsu, 1974; Elder & Alexander, 1982). Among the endoglycosidases, three of the most useful are endoglycosidase H, endoglycosidase D, and endoglycosidase F which promote the cleavage of high mannose, complex glycans, and both types of glycans, respectively, from glycoproteins and glycopeptides. Cleavage occurs at the glycosidic linkage adjacent to the *N*-acetylglucosamine which links the carbohydrate chain to the protein asparagine residue, thus leaving the *N*-acetylglucosamine residue bound to the protein. More recently an endoglycosidase classified as an endoglycopeptidase was found in almond emulsin (Takahashi, 1977; Takahashi & Nishibe, 1978) and was partially purified (Takahashi & Nishibe, 1981; Plummer & Tarentino, 1981; Tarentino & Plummer, 1982). The major difference between these two classes of enzyme is that the endoglycopeptidases from almond appear to have a broader specificity, cleaving glycoproteins and glycopeptides with high mannose and complex types of glycans. Moreover, the endoglycopeptidases hydrolyze the β-aspartylglycosylamine linkage, freeing the intact carbohydrate chain and resulting in a protein backbone having an additional aspartic acid residue.

A major hindrance in the study of such enzymes has been the cumbersome nature of the assays required. The procedure described here to assay the endoglycopeptidase activity is both rapid and convenient. It is based upon solubility of the oligosaccharide but not the intact glycopeptide in cold aqueous ethanol, and consequently the assay does not require the use of time-consuming manipulations such as electrophoresis or thin-layer chromatography to assay each fraction, nor does it require the synthesis of labeled substrate.

Enzyme purification by one early procedure involved extraction, desalting, two ion-exchange chromatography steps, one gel filtration chromatography step, and preparative polyacrylamide gel electrophoresis to obtain a final enzyme preparation which was purified more than 700-fold and with a yield of 63%. Because we found that the endoglycosidase from almond emulsin is itself a glycoprotein, we were able to exploit this property of the enzyme in order to develop a more efficient procedure to purify quantities of the enzyme. This procedure involves extraction, two ion-exchange column chromatography steps, an affinity chromatography step, and one gel filtration chromatography step (Table I). By use of such a procedure, homogeneous enzyme can be obtained in a week in a yield of 37% and with a purification factor of approximately 1200-fold, starting with commercial samples of β-glucosidase or starting with almond meal as an inexpensive source, in which case homogeneous enzyme was obtained with a recovery of 45% and a purification factor of 9000-fold.

During the course of the purification, we found that the endoglycopeptidase copurified with part of the initial β-hexosaminidase activity, and the two activities could be separated only with difficulty. The apparent contamination of endoglycopeptidase with β-hexosaminidase has been reported (Takahashi & Nishibe, 1981; Tarentino & Plummer, 1982). These authors attributed the contamination of the enzyme to differences in lots of almond emulsin. In all of our starting preparations we find that there is a large amount of β-hexosaminidase, due to one or more distinct hexosaminidases. The kinetic and molecular properties of the peptide *N*-glycosidase are different from those of jack bean β-hexosaminidase (Li & Li, 1979). In contrast to results obtained by SDS–polyacrylamide gel electrophoresis for the β-hexosaminidases from human placenta (Hasilik & Neufeld, 1980), kidney (Srivastava et al., 1976), and liver (Srivastava & Beutler, 1974), all of which show those enzymes to be composed of several subunits, the present enzyme consists of a single polypeptide. The present study suggests that the β-hexosaminidase activity is a contamination. Although not consistent with the thermal denaturation and inactivation studies, where both activities disappear simultaneously, it does seem consistent with the differing K_i values for competitive inhibition, and with the very low hexosaminidase activities observed after affinity chromatography (Table I).

The purified enzyme is homogeneous by polyacrylamide gel electrophoresis under native conditions at pH 8.9 and pH 4.5, and also on SDS–polyacrylamide gel electrophoresis, at protein concentrations as high as 15 μg of protein per gel. The same band is also stained by Schiff's reagent, indicating that the glycopeptidase is itself a glycoprotein. The results obtained with SDS–polyacrylamide gel electrophoresis show that the enzyme has a molecular weight of 66 800. A molecular weight of 58 896 is obtained by integer fit of the amino acid composition data. The only previous estimate of a molecular weight reported for such an enzymatic activity is 80 × 10^3, by gel filtration chromatography on Sephacryl S-200 (Tarentino & Plummer, 1982). We observe that the enzyme is eluted from Sephacryl S-200 at an elution volume which corresponds to a molecular weight of 90 000. These differences can be attributed to the presence of carbohydrate in the glycopeptidase molecule. The presence of large amounts of glucosamine in the amino acid analyses, a positive Schiff reaction shown by this protein even after SDS gel electrophoresis, and its retention on concanavalin A chromatography all supported this conclusion. This was confirmed by a direct gas chromatographic analysis of the carbohydrate content of the homogeneous enzyme (Table II).

The isoelectric point of the enzyme is between 7.0 and 7.7. On isoelectric focusing the homogeneous enzyme displays a broad band in this region indicating substantial charge heterogeneity that is possibly caused by the presence of modified carbohydrate residues in the enzyme molecule. A somewhat similar p*I* of 7.7–8.7 has been reported for the crude enzyme (Takahashi & Nishibe, 1981). These authors describe the apparent presence of three isoenzymes with differing specificities toward glycopeptides and glycoproteins.

When ovomucoid glycopeptide is used as an assay substrate, the homogeneous enzyme possesses a broad pH optimum around pH 4–6, with the enzyme retaining 30% of activity at pH 3 but no activity at pH 7.5. Maximal activity was observed near pH 4.5 which is consistent with that reported (Tarentino & Plummer, 1982) for a partially purified fraction.

The enzyme completely removes the carbohydrate from some glycopeptides. This is also consistent with high-pressure liquid chromatography experiments (Figure 5), where we observe a progressive disappearance of the glycopeptide peak accompanied by the appearance of peaks due to the low molecular weight peptide and to the oligosaccharide. This pro-

vides the basis for another rapid quantitative assay. Takahashi et al. (1982) were also able to separate glycosylated and deglycosylated takaamylase A, but that system was unsuitable for quantitative work.

Comparison of the apparent rates of hydrolysis of complex and high mannose glycopeptides suggests that the type and size of the oligosaccharide as well as peptide chain length may affect the enzyme action. An effect of peptide chain length and the position of the carbohydrate asparagine link within the protein on the hydrolysis rate has been documented by Plummer & Tarentino (1981). When we compare the rate of hydrolysis of ovomucoid and GP158 glycopeptides, which possess complex glycans of different sizes but which contain similarly sized peptides of 8 and 11 amino acid residues, respectively, we observe a significant difference in hydrolysis rates of the glycopeptides (Figure 6). This result suggests that the marked differences in the rate of hydrolysis of the glycopeptides may be due to differences in the size of the carbohydrate chains, an effect which has been only briefly considered (Plummer & Tarentino, 1981).

Takahashi et al. (1982) and Takahashi & Nishibe (1981) were able to cleave the carbohydrate from some native proteins such as stem bromelain, ovalbumin, ovotransferrin, desialofibrinogen, and takaamylase A and the derived glycopeptides, but Tarentino & Plummer (1982) stated that they were able to cleave carbohydrate from glycoproteins only in the presence of chaotropic salts or when the protein was heat denatured in the presence of SDS. They also concluded that the presence of β-mercaptoethanol in the reaction mixture is essential to achieve a complete removal of glycans from glycoproteins. With these differences in mind and also considering that the endoglycopeptidase did not cleave the carbohydrate from native turkey ovomucoid, we conducted a systematic study of the enzyme stability and activity in the presence of known protein denaturants. Our results show that this enzyme is stable under a wide range of conditions including the presence of high concentrations of urea, Triton X-100, Tween 80, Nonidet P-40, β-mercaptoethanol, LiCl, cationic detergents, Zwittergent, and EDTA. Consistent with the results of Takahashi & Nishibe (1981), the homogeneous enzyme is not inhibited by EDTA or by protease inhibitors. The presence of divalent cations such as Zn^{2+} and Co^{2+} can cause a modest (40%) increase in enzyme activity. Readily dissociable metal ions are apparently not required for enzymatic activity. However, the enzyme is highly sensitive to the presence of SDS, and concentrations as small as 0.01% inactivate the enzyme. Among other chemicals tested only guanidine hydrochloride partially inactivated the enzyme (at a concentration of 1.5 M). Even though the presence of 0.01% SDS or 1.5 M guanidine hydrochloride causes a loss of enzymatic activity, the present fluorescence and circular dichroism spectral studies indicate that the overall conformation of the almond endoglycosidase is not detectably altered.

Employing experimental conditions based on these findings, we were then able to cleave the carbohydrate from native ovomucoid in the presence of Triton X-100, Tween 80, urea, KSCN, $NaClO_4$, SDS, Zwittergent, guanidine hydrochloride, LiCl, and β-mercaptoethanol. However, this behavior is not general. Using standard conditions in the presence of urea or of mercaptoethanol, we surveyed the ability of the enzyme to cleave the carbohydrate from a variety of proteins. Of those tested, the carbohydrate was readily hydrolyzed from the turkey ovomucoid and from ribonuclease B, but no reaction was observed with ovalbumin, orosomucoid, or human prostatic acid phosphatase. Consequently, it appears that the action of this enzyme is rather different in relation to each glycoprotein, and the optimal hydrolysis conditions may have to be sought for each particular glycoprotein. Most importantly, the ready availability of a homogeneous, well-characterized peptide *N*-glycosidase and a convenient new assay will greatly facilitate work with this important enzyme.

Acknowledgments

We thank Dr. Haile Mehansho for a gift of glycopeptide GP158, Drs. Paul Weber and Bradley Sheares for the carbohydrate analyses, and James Cook and Prof. Michael Laskowski for amino acid and HPLC analyses.

Registry No. Peptide *N*-glycosidase, 83534-39-8.

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