Purification and Characterization of Endo- β -N-acetylglucosaminidase from a *Flavobacterium* sp.

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A gram negative bacterium isolated from soil was found to produce a high level of endo- β -N-acetylglucosaminidase in the culture medium. The organism was identified as a *Flavobacterium* sp. from various bacteriological characteristics. The enzyme from the *Flavobacterium* sp. was purified to homogeneity from culture broth by fractionation with ammonium sulfate and column chromatographies on DEAE-cellulose, hydroxylapatite, and Sephadex G-150 and G-100. The molecular weight of the enzyme was estimated to be 27,000 and 30,000 by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively, and it appeared to consist of a single polypeptide chain. The optimal pH for activity was 5.0 to 6.0 and the stable pH range was 5~7. The Michaelis constant was 0.30 mM with dansyl-Asn-(GlcNAc)₂(Man)₆ as the substrate. The enzyme hydrolyzed oligosaccharides of native ovalbumin, bovine pancreatic ribonuclease B and a yeast invertase.

Endo- β -N-acetylglucosaminidase (EC 3.2.1.96) hydrolytically cleaves the N, N'-di-acetylchitobiose moiety in the core region of asparagine-linked oligosaccharides of various glycoproteins.¹⁾

Because of its characteristics, endo- β -N-acetylglucosaminidase is becoming an indispensable tool for studies on glycoprotein structure and function. The purification of this enzyme has been reported from various sources such as *Streptomyces plicatus* (the enzymes were named Endo-H and Endo-L),^{2,3)} *Diplococcus pneumonia* (named Endo-D),⁴⁾ *Clostridium perfringens* (named Endo-C_I and Endo-C_{II}),⁵⁾ *Flavobacterium meningosepticum* (named Endo-F),⁶⁾ *Bacillus circulans*,⁷⁾ *Bacillus alvei*,⁸⁾ a basidiomycete,⁹⁾ fig latex¹⁰⁾ and hen oviduct.¹¹⁾ Among them only Endo-H²⁾ and

Endo-D¹²⁾ were purified to homogeneity from cultural filtrates of *S. plicatus* and *D. pneumonia*, respectively.

Four enzymes, endo- β -N-acetylglucosaminidases H, D, C_I and C_{II}, have been extensively investigated as to their enzymatic action spectra on various glycopeptides, and they seem to have different strict specificities as to the oligomannosyl structures of their substrates.^{13~18)} Although they have proven useful, it is difficult to obtain the pure enzymes in sufficient amounts. Because they are anaerobic pathogenic bacteria, the use of *D. pneumonia* and *Cl. perfringens* is difficult, and the yields of endo- β -N-acetylglucosaminidases with them are very poor. Purification of the enzyme from the culture filtrate of *S. plicatus* is rather complicated. Thus, we searched for a better source of endo- β -N-acetylglucosaminidase and found that a bacterial strain isolated from a soil sample produced a very high level of endo- β -

Abbreviations: GlcNAc, N-acetylglucosamine; Asn, asparagine; DNS, dansyl (5-dimethylaminonaphthalene-1-sulfonyl).

N-acetylglucosaminidase in the culture medium. Moreover, we purified the enzyme by a comparatively simple procedure.

In this paper, we describe the identification of a bacterium that produces endo- β -*N*-acetylglucosaminidase, and the purification and properties of the endo- β -*N*-acetylglucosaminidase.

MATERIALS AND METHODS

Microorganism and cultivation. A bacterium, strain No. 1022, which was isolated from soil in Kyoto city was used throughout this study. The bacterium was identified as a strain of the genus *Flavobacterium* as described under RESULTS. The maintenance medium contained 0.5% glucose, 0.5% peptone, 1.0% yeast extract, 0.5% NaCl and 2% agar. The pH was adjusted to pH 7.0. This medium was also used for liquid cultures. The bacterium was transferred from a slant culture to 5 ml of the medium in a test tube, and the seed culture was performed at 28°C for 28 hr with shaking. Each culture was then transferred to a 2-liter shake flask containing 500 ml of the medium, and cultivation was carried out at 28°C on a reciprocal shaker.

Preparation of substrates. Dansyl asparagine oligosaccharides (DNS-glycopeptides) as substrates for the enzyme assay were prepared as follows: the purification of ovalbumin from egg whites was performed on a CM-cellulose column according to the method of Rhodes *et al.*¹⁹⁾ Ovalbumin glycopeptide was prepared by pronase digestion of purified ovalbumin followed by separation on Sephadex G-25 gel by the procedure of Huang *et al.*²⁰⁾ Dansyl derivatives of ovalbumin glycopeptides were prepared according to the method of Gray,²¹⁾ followed by paper chromatography to separate dansylsulfonic acid and dansyl asparagine oligosaccharides. The dansyl asparagine oligosaccharides which were eluted with water contained every heterogeneous carbohydrate chain in ovalbumin (there are five different carbohydrates of ovalbumin, which comprise various proportions of D-mannose and GlcNAc²²⁾), which was determined by reverse-phase high-performance liquid chromatography (HPLC) by Dr. H. Iwase, Kitasato University.²³⁾

Materials. Hydroxylapatite was prepared according to the method of Tiselius *et al.*²⁴⁾ Sephadex G-200, G-150, G-100 and G-25, and Concanavalin A-Sepharose 4B were purchased from Pharmacia Fine Chemicals Co. PBE 94 gel and Polybuffer 74-HCl for chromatofocusing, and molecular weight markers were also obtained from Pharmacia Fine Chemicals Co. Ribonuclease B (from bovine pancreas), invertase (from baker's yeast) and yeast mannan were purchased from Sigma Chemical Co. The ovalbumin glycopeptide IV, (Man)₆(GlcNAc)₂Asn, was kindly do-

nated by Dr. H. Iwase, Kitasato University.

Enzyme assay. The reaction mixture was composed of 50 nmol of DNS-glycopeptides, 2 μ mol of potassium phosphate buffer (pH 6.0) and the enzyme solution in a total volume of 35 μ l. After incubation for an appropriate time at 37°C, the reaction was terminated by the addition of 5 μ l of 40% trichloroacetic acid. An aliquot of the reaction mixture was applied to a filter paper (20 cm square, Toyo Filter Paper No.51A), and paper chromatography was performed by the ascending technique using a solvent system of *n*-butyl alcohol-acetic acid-water (3:1:1). The product, DNS-Asn-GlcNAc, was located from its strong yellow orange fluorescence on exposure to ultraviolet light. The spot corresponding to DNS-Asn-GlcNAc was cut out and extracted with 3 ml of water, and its amount was determined from the fluorescence (excitation, 313 nm; emission, 540 nm) with a Hitachi fluorescence spectrophotometer MPF-2A.²³⁾ One unit is defined as the amount of enzyme that yields 1 μ mol of DNS-Asn-GlcNAc per min at 37°C under the assay conditions.

Exoglycosidases were assayed with the appropriate *p*-nitrophenyl glycosides (such as *p*-nitrophenyl α -mannoside, β -GlcNAc, *etc.*). Protease activity was assayed with azocoll.²⁵⁾

Analytical methods. Protein was determined by the method of Lowry *et al.*²⁶⁾ with crystalline egg albumin as the standard. The protein elution patterns on column chromatography were determined on the basis of the absorption at 280 nm. The percent extinction coefficient of the purified endo- β -*N*-acetylglucosaminidase, $E_{1\text{cm}}^{1\%}$ at 280 nm, was 15.5. Neutral sugars were assayed by the phenol-H₂SO₄ reaction.²⁷⁾ Ultracentrifugal analysis was performed with a Spinco model E ultracentrifuge at 60,000 rpm at 20°C. The sedimentation coefficient was obtained with the enzyme concentrations of 0.58, 0.39 and 0.30%.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Davis²⁸⁾ using 7.5% polyacrylamide gel with Tris-glycine buffer, pH 8.3. Electrophoresis was carried out at a constant current of 2 mA per column. Sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis was performed in 12.5% acrylamide and 0.1% SDS with a discontinuous Tris-glycine buffer system according to the method of Laemmli.²⁹⁾ The molecular weights of proteins were determined after calibration with the following standards: α -lactalbumin (MW, 14,400), soybean trypsin inhibitor (MW, 20,100), carbonic anhydrase (MW, 30,000), ovalbumin (MW, 43,000), bovine serum albumin (MW, 67,000) and phosphorylase *b* (MW, 94,000). Protein and carbohydrate were stained with Coomassie Brilliant Blue R and by the fuchsin-periodate method,³⁰⁾ respectively.

Molecular weight determination. The molecular weight of the enzyme was determined by gel filtration on Sephadex G-200 according to the method of Andrews.³¹⁾ Elution was carried out with 10 mM potassium phosphate buffer (pH 7.0). The following proteins were employed as molecular weight markers: ribonuclease (MW, 13,700), chymotrypsinogen A (MW, 25,000), ovalbumin (MW, 43,000) and bovine serum albumin (MW, 67,000).

Gel chromatofocusing. Gel chromatofocusing³²⁾ was performed to determine the isoelectric point of the enzyme. The enzyme was applied to a PBE 94 gel column (1.1 $\times$ 13.5 cm) previously equilibrated with 0.025 M imidazole-HCl buffer (pH 7.4). Chromatofocusing was performed by elution with Polybuffer-HCl (pH 4.0).

Amino acid analysis. The enzyme protein was hydrolyzed with 6 N HCl at 110°C for 24 hr. Then, analysis of amino acids in the hydrolyzates was carried out according to the method of Spackman *et al.*³³⁾ with a Kyowa amino acid analyzer K-101. Cysteine and half cystine were determined from the amount of cysteic acid as described by Moore.³⁴⁾ Tryptophan was determined spectrophotometrically as described by Goodwin and Morgan.³⁵⁾

Scanning electron microscopy. Scanning electron microscopy was performed with a Akashi alpha-9 Electron Microscope. The cell pastes were fixed in 1% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.0)–0.15 M NaCl solution for 2 hr. The fixed cells were dehydrated in a graded acetone series and then put in isoamyl alcohol. Specimens were then subjected to critical point drying and finally coated with gold-palladium.

RESULTS

Identification of strain No.1022

Microbiological properties of the bacterium (strain No.1022) were investigated according to the methods and procedures described in the "Manual of Methods for General Bacteriology."³⁶⁾ The results are shown in Table I. From these results and with reference to "Bergey's Manual of Determinative Bacteriology, 8th Edition,"³⁷⁾ the strain was identified as a strain of *Flavobacterium*. A scanning electron micrograph of the strain is shown in Fig. 1.

Production of endo- β -N-acetylglucosaminidase

The enzyme production in the supernatant at different stages of growth was investigated. As shown in Fig. 2, the cell growth reached the

TABLE I. BACTERIOLOGICAL CHARACTERISTICS OF STRAIN No. 1022

Morphological	
Cells:	rods (0.4~0.5 $\times$ 1.2~1.5 μ m) with rounded ends
Mobility:	non-motile, lacking flagella
Gram-stain:	negative
Physiological	
Aerobic:	produced insoluble yellow pigment
O-F test:	oxidative
Nitrate reduction:	negative
Catalase:	produced
Oxidase:	produced
Indole:	not produced
Gelatin and casein:	hydrolyzed
Starch:	not hydrolyzed
Litmus milk:	acid, digested
Citrate:	not utilized
MR test:	negative
Acid production without gas from glucose	
No growth at 5°C, 42°C

FIG. 1. Scanning Electron Micrograph of the New *Flavobacterium* sp.

The bar length is 1 μ m.

maximum level in one day, whereas the enzyme activity was first detected after 2 days-cultivation and was highest after 4~5 days when autolysis began to take place.

Purification of endo- β -N-acetylglucosaminidase

All procedures were carried out at 0~5°C. Potassium phosphate buffer (pH 7.0) was used throughout the purification.

Crude enzyme in culture fluid. After 4 days'

FIG. 2. Time-Courses of Growth and Enzyme Production in a Culture of the New *Flavobacterium* sp.

The culture was carried out in a 2-liter shake flask containing 500 ml of medium at 28°C on a reciprocal shaker.

○, Endo- β -N-acetylglucosaminidase; ●, cell growth; ▲, pH.

cultivation, 9.5 liters of clear culture fluid was obtained after centrifugation at $10,000 \times g$ for 30 min. The supernatant was used as the crude enzyme preparation for the purification. No exoglycosidase or protease activities could be detected in the supernatant.

Ammonium sulfate fractionation. The supernatant was brought to 90% saturation with ammonium sulfate and then stood overnight. The resulting precipitate was collected by centrifugation and dissolved in a minimal volume of 10 mM phosphate buffer, followed by dialysis against the same buffer overnight.

DEAE-cellulose column chromatography. The dialyzed solution was applied to a DEAE-cellulose column (6.0×45 cm) equilibrated with 10 mM phosphate buffer. After the column had been washed with the same buffer, the enzyme was eluted with the buffer containing 0.1 M NaCl. The active fractions were collected and concentrated by Amicon ultrafiltration with a PM-10 membrane. The concentrate was dialyzed against 1 mM phosphate buffer overnight.

Hydroxylapatite column chromatography. The dialyzed enzyme solution was applied to a hydroxylapatite column (2.6×24 cm) previously equilibrated with 1 mM phosphate buffer. The column was washed with the same buffer. The enzyme was eluted with 10 mM phosphate buffer. The active fractions were

FIG. 3. Elution Profile of the Enzyme on a Sephadex G-100 Column.

The enzyme was applied to a column (2.0×113 cm) of Sephadex G-100 equilibrated with 10 mM potassium phosphate buffer (pH 7.0). Elution of the enzyme was performed with the same buffer.

○, Endo- β -N-acetylglucosaminidase; -----, absorbance at 280 nm.

pooled and concentrated by ultrafiltration.

Sephadex G-150 column chromatography. The concentrated enzyme solution was applied to a Sephadex G-150 column (2.0×113 cm) equilibrated with 10 mM phosphate buffer. The enzyme was eluted with the same buffer, and the active fractions were collected and concentrated by ultrafiltration.

Sephadex G-100 column chromatography. The concentrated enzyme was placed on the top of a column (2.0×113 cm) of Sephadex G-100 previously equilibrated with 10 mM phosphate buffer and eluted with the same buffer. The enzyme fractionation profile is shown in Fig. 3. The fractions containing the enzyme were pooled and concentrated by ultrafiltration.

The results of the enzyme purification are summarized in Table II. The enzyme was purified about 1,140-fold with a recovery of about 30%.

Enzyme purity

The purified enzyme migrated as a single protein band on both polyacrylamide disc gel and SDS-polyacrylamide gel electrophoresis, as shown in Fig. 4. In addition, it showed a

TABLE II. PURIFICATION OF ENDO- β -N-ACETYLGLUCOSAMINIDASE FROM A *Flavobacterium* sp.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture fluid	36,400	1,290	0.035	100
Ammonium sulfate precipitation	2,120	1,230	0.58	95
DEAE-cellulose column chromatography	84.2	931	11.1	72
Hydroxylapatite column chromatography	33.4	698	20.9	54
Sephadex G-150 column chromatography	15.4	578	37.5	45
Sephadex G-100 column chromatography	10.3	411	39.9	32

FIG. 4. Polyacrylamide Gel Electrophoresis of the Purified Enzyme.

(A) Polyacrylamide disc gel electrophoresis.

The purified enzyme was applied to a 7.5% polyacrylamide gel.

(B) SDS-polyacrylamide slab gel electrophoresis.

The purified enzyme was subjected to SDS-polyacrylamide slab gel electrophoresis with 10% acrylamide. The protein standards used were: (1) α -lactalbumin (MW, 14,400), (2) soybean trypsin inhibitor (MW, 20,100) (3) carbonic anhydrase (MW, 30,000), (4) ovalbumin (MW, 43,000), (5) bovine serum albumin (MW, 67,000) and (6) phosphor-ylase *b* (MW, 94,000). a, 15 μ g of enzyme; b, 30 μ g of enzyme; st, standard proteins.

single and symmetric peak in the sedimentation pattern on analytical ultracentrifugation. The ultracentrifugation revealed that the purified enzyme had a sedimentation coefficient of 2.3 *S*.

TABLE III. AMINO ACID COMPOSITION OF ENDO- β -N-ACETYLGLUCOSAMINIDASE

The molar contents were calculated on the basis of a molecular weight of 30,000.

	Residues/mol of protein
Aspartic acid	28
Threonine	14
Serine	19
Glutamic acid	16
Proline	6
Glycine	23
Alanine	26
Valine	14
Methionine	3
Isoleucine	10
Leucine	15
Tyrosine	12
Phenylalanine	7
Lysine	9
Histidine	3
Arginine	4
Tryptophan	20
Half cystine	2

Molecular weight and isoelectric point

The molecular weight of the enzyme was estimated to be 27,000 by gel filtration. SDS-polyacrylamide gel electrophoresis gave a single protein band corresponding to a molecular weight of 30,000 (Fig. 4). These results indicate that the enzyme has a monomeric structure.

The isoelectric point of the enzyme was found to be 4.5 on chromatofocusing.

FIG. 5. Effects of pH on the Activity and Stability of the Enzyme.

(a) The enzyme was assayed in 0.05 M buffers of various pHs under the standard conditions.

(b) The enzyme was pre-treated in 0.5 M buffers of different pHs at 4°C for 2 days and then the remaining enzyme activity was assayed at pH 6.0.

●, sodium citrate-HCl; ○, sodium acetate-acetic acid; ●, potassium phosphate; ○, Tris-HCl; ●, glycine-NaOH.

Amino acid composition

The amino acid composition of the enzyme is shown in Table III. It seemed that the enzyme had relatively low contents of arginine and cysteine.

Effects of pH and temperature on the activity and stability

The optimum pH of the enzyme was found to be 5.0 to 6.0 (Fig. 5a). The enzyme was stable in the pH range of 5~7 when kept at 4°C for 2 days (Fig. 5b). The enzyme showed the highest activity at 50°C under the standard assay conditions. Its thermal stability was examined by heating it at various temperatures for 10 min. The enzyme was found to be stable up to 45°C. It is noteworthy that the enzyme still showed about 35% of the original activity after incubation at 80°C for 10 min.

Effects of various compounds

The effects of metal ions and SH-reagents on the enzyme activity were examined by incubating the enzyme with the compounds at 37°C and then directly assaying the activity. The activity of the enzyme was not significantly affected by the following compounds at 1 mM: CaCl_2 , CoCl_2 , MgSO_4 , MnSO_4 , CuSO_4 , ZnSO_4 , FeSO_4 , EDTA, *p*-chloromercuric

FIG. 6. Sephadex G-100 Chromatography of the Products Formed from Native Ovalbumin by the Enzyme.

Ovalbumin (20 mg) was incubated in 100 mM acetate buffer (pH 5.0) at 37°C for the indicated times. Aliquots were analyzed for hexose (●) from the phenol- H_2SO_4 reaction (the absorbance at 490 nm) and for protein (-----) from the absorbance at 280 nm.

benzoate, *N*-ethylmaleimide, dithiothreitol, 2-mercaptoethanol and glutathione. Only HgCl_2 inactivated the enzyme significantly, to 15% of the original activity.

The effect of various sugars on the enzyme activity were also investigated. The following sugars had little or no effect on the enzyme activity: 10 mM glucose, galactose, glucosamine, galactosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine, diacetylchitobiose and triacetylchitotriose. The enzyme was not inhibited by 50 mM mannose or 15 mg/ml of yeast mannan.

Effect of substrate concentration

The effect of the substrate concentration on the initial velocity of the enzyme reaction was examined at 37°C using DNS-glycopeptides. The apparent Michaelis constant (K_m) was 0.40 mM. For DNS-GP-IV, DNS-Asn-(GlcNAc) $_2$ (Man) $_6$, which is one of the dansylated compounds of the ovalbumin glycopeptides, the K_m was 0.30 mM.

Release of sugar chains from intact glycoproteins.

The purified enzyme was tested as to its substrate specificity for oligosaccharides derived from various glycoproteins such as ovalbumin, ribonuclease B and yeast invertase. Ovalbumin (20 mg) was incubated with 19

FIG. 7. Concanavalin A-Sepharose 4B Chromatography of the Products Formed from Native Ovalbumin by the Enzyme.

The products (about 7 mg) formed from native ovalbumin by the enzyme were applied to a Concanavalin A-Sepharose 4B column equilibrated with 10 mM potassium phosphate buffer (pH 7.0) and eluted with the same buffer. An arrow indicates changing of the effluent to 0.25 M methyl α -mannoside in the same buffer.

units of the enzyme in 100 mM acetate buffer, pH 5.0, in a total volume of 1.0 ml at 37°C. After 48 hr the reaction was terminated by the addition of HgCl_2 , and the digest was chromatographed on a column of Sephadex G-100 (0.8 $\times$ 114 cm) with 10 mM potassium phosphate buffer, pH 7.0, in order to separate the protein and the oligosaccharide products from ovalbumin (Fig. 6). Prior to treatment with the enzyme, the carbohydrate and protein peaks coincided. After the treatment, however, a new separate peak of carbohydrate was found behind the peak corresponding to undigested ovalbumin. The digest was also applied to a Concanavalin A-Sepharose 4B column (0.8 $\times$ 20 cm), which was eluted with 0.01 M potassium phosphate buffer (pH 7.0) followed by with the same buffer containing 0.25 M methyl α -mannoside. As shown in Fig. 7, the carbohydrate-depleted protein was unadsorbed, and undigested ovalbumin was eluted with the buffer containing methyl α -mannoside. Carbohydrate associated with native ovalbumin was detected by the Schiff-periodate color reaction after SDS polyacrylamide slab gel electrophoresis, whereas no color was detected with the pooled oligosaccharide-depleted protein fraction solution.

The purified enzyme could also hydrolyze the oligosaccharides of bovine ribonuclease B

and yeast invertase. After treatment of the ribonuclease (10 mg) with 19 units of the enzyme at pH 6.0 for 25 hr, the oligosaccharide released was obtained by Sephadex G-100 column gel filtration. On the other hand, the treatment of yeast invertase (2 mg) with 6 units of the enzyme at pH 5.0 for 23 hr resulted in complete removal of the oligosaccharides, which was shown by SDS polyacrylamide gel electrophoresis.

DISCUSSION

During a search for useful glycosidases in the culture fluids of various microorganisms, we found a bacterial strain that produced the highest level of endo- β -N-acetylglucosaminidase among many microorganisms isolated from soils. The strain was identified as a *Flavobacterium* sp., and seemed to be a different species from *F. meningosepticum* which produces the Endo-F enzyme,⁶⁾ with respect to some microbiological properties. For example, the new *Flavobacterium* sp. produces a bright yellow pigment but not indole. On the other hand, *F. meningosepticum* is frequently nonpigmented and, when a yellow pigment is encountered, the degree of pigmentation is very slight, and the strain produces indole. Moreover, the endo- β -N-acetylglucosaminidase activity of the newly isolated *Flavobacterium* sp. is more than 300-fold higher than that of *F. meningosepticum* (data not shown). This organism is aerobic and thus easier to maintain and culture than *D. pneumoniae* and *Cl. perfringens*, which produce the Endo-D and Endo-C_I, C_{II} enzymes, respectively.

This *Flavobacterium* sp. endo- β -N-acetylglucosaminidase was purified to homogeneity by a comparatively simple procedure with a high recovery of the total activity in comparison with the Endo-H enzyme of *S. plicatus*,²⁾ although the two enzymes have some similar properties such as molecular weight, optimum pH and stability. Actually, there were no exoglycosidase or protease activities in the culture fluid of the new *Flavobac-*

terium sp., and this seems to be advantageous for purification of the enzyme. Moreover, the specific activity of the enzyme in the culture fluid was the highest reported previously for any microorganism, when the GP-IV oligosaccharide, (Man)₆(GlcNAc)₂-Asn, of ovalbumin was used as the substrate for the assay.

Although its strict substrate specificity is still obscure, the endo- β -*N*-acetylglucosaminidase of the new *Flavobacterium* sp. seems to act on both high-mannose and hybrid types of oligosaccharides of ovalbumin glycopeptides according to the results of HPLC analysis using DNS-ovalbumin glycopeptides (data not shown). Furthermore, the enzyme could release oligosaccharides from native ovalbumin, bovine pancreatic ribonuclease B and an invertase from yeast. Though the Endo-H enzyme cannot hydrolyze native ovalbumin,¹³⁾ the new *Flavobacterium* sp. enzyme could act on it and release sugar chains, which was shown by column chromatographies on Sephadex G-100 gel and Con A-Sepharose 4B. This paper is the first report of an endo- β -*N*-acetylglucosaminidase that releases sugar chains from native ovalbumin.

The enzyme was not affected by any SH-reagent other than HgCl₂. This seems to be due to the presence of only 2 cysteine residues in the enzyme molecule. The enzyme was also not inhibited by the presence of mannose or yeast mannan, which is different from in the cases of the Endo-D and Endo-H enzymes,³⁸⁾ and this is beneficial for its use as an analytical tool.

The specificities of the enzymes from *D. pneumoniae* and *S. plucatus* are extremely different with respect to the structure of the oligomannosyl cores of their substrates. The Endo-D enzyme generally does not hydrolyze glycopeptides of mannose-*N*-acetylglucosamine units, except Asn-(GlcNAc)₂-(Man)₅,¹⁵⁾ while the Endo-H enzyme hydrolyzes various carbohydrate chains composed of the unit.¹⁵⁾ All of the microbial enzymes except the C₁ enzyme have similar substrate specificities to that of the Endo-H

enzyme. The new *Flavobacterium* sp. enzyme seems to have a similar substrate specificity to that of other microbial enzymes from *Streptomyces*, *Clostridium* and *Bacillus*.

The substrate specificity of and a means of obtaining a sufficient amount of endo- β -*N*-acetylglucosaminidase of the new *Flavobacterium* sp. are currently being investigated.

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