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Induction and Purification of Endo-β-*N*-Acetylglucosaminidase from *Arthrobacter protophormiae* Grown in Ovalbumin

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Arthrobacter protophormiae **produced a high level of extracellular endo-β-*N*-acetylglucosaminidase when cells were grown in a medium containing ovalbumin. The enzyme was induced by the glycopeptide fraction of ovalbumin prepared by pronase digestion. Production of the enzyme was also induced by glycoproteins such as yeast invertase and bovine ribonuclease B but not by monosaccharides such as mannose, *N*-acetylglucosamine, and galactose. The enzyme was purified to homogeneity as demonstrated by polyacrylamide gel electrophoresis and has an apparent molecular weight of about 80,000. The enzyme showed a broad optimum pH in the range of pH 5.0 to 11.0. The enzyme hydrolyzed all heterogeneous ovalbumin glycopeptides, although the hydrolysis rates for hybrid type glycopeptides were very low. The substrate specificity of *A. protophormiae* endo-β-*N*-acetylglucosaminidase was very similar to that of Endo-C_{II} from *Clostridium perfringens*. Therefore, the enzyme induction by *A. protophormiae* seems to have a close relation to the substrate specificity of the enzyme.**

Endo-β-*N*-acetylglucosaminidase (endo-β-GlcNAc-ase, EC 3.2.1.96) hydrolytically cleaves the *N,N'*-diacetylchitobiose moiety of asparagine-linked sugar chains of various glycoproteins. The purification of this enzyme has been reported from some microorganisms, and the substrate specificities of the enzyme have been elucidated (5, 9, 13, 34). Because of its characteristics, endo-β-GlcNAc-ase has been used as an indispensable tool for structural studies on glycoproteins.

Endo-β-GlcNAc-ase is also found in animal tissues, and the enzyme in human tissues plays an important role in the catabolism of asparagine-linked sugar chains of glycoproteins (28). Although many microorganisms produce endo-β-GlcNAc-ase, no studies on the physiological roles of the enzyme in microorganisms have been described to date. Recently, we reported that a component of yeast cells remarkably induced the production of endo-β-GlcNAc-ase from a *Flavobacterium* sp. isolated from soil (12). Therefore, we investigated culture conditions favoring the production of microbial endo-β-GlcNAc-ase. Then we found that *Arthrobacter protophormiae* highly induced endo-β-GlcNAc-ase in culture fluid when grown in medium containing ovalbumin.

In this report, we describe the induction of an extracellular endo-β-GlcNAc-ase from *A. protophormiae* grown on various glycoproteins. We also report the purification of the enzyme and its substrate specificities toward various asparagine-linked oligosaccharides.

MATERIALS AND METHODS

Chemicals. DEAE-Toyopearl 650M was purchased from Tosoh Co., Tokyo, Japan. Molecular weight markers were obtained from Pharmacia Fine Chemical Co. (Piscataway, N.J.). Pronase and dansyl (dns) chloride were purchased from Kakenkagaku Co. and Tokyo Kasei Co., respectively. Bovine serum albumin, bovine RNase B, yeast invertase, human transferrin, yeast mannan, and azocasein were purchased from Sigma Chemical Co. (St. Louis, Mo.). Pressed baker's yeast was obtained from the Oriental Yeast Co. Di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose were kindly donated by Katokichi Co. Ltd., Japan. Colloidal chitin was prepared from commercial chitin (Wako Pure Chemical Co.) by the method of Berger and Reynolds (2). *p*-Nitrophenyl glycosides were purchased from Seikagaku Kogyo Co. All other chemicals were of analytical reagent grade.

Microorganisms and cultivation. *A. protophormiae* (AKU 0647) was used throughout this investigation (*Brevibacterium protophormiae* was recently reclassified as *A. protophormiae*) (27). The bacterium was cultured in a liquid medium composed of 0.5% peptone, 0.5% yeast extract, 0.5% NaCl, and 1.0% lyophilized ovalbumin powder, pH 7.5. A loopful of cells was inoculated into 5 ml of the medium in a test tube, and the seed culture was grown at 28°C for 18 h with shaking. The culture was then transferred to a 500-ml Erlenmeyer flask containing 150 ml of the medium, and cultivation was continued at 28°C on a reciprocal shaker.

Preparation of substrates. Ovalbumin was prepared from egg whites by the method of Rhodes et al. (24). Glycopeptides (GP) from both ovalbumin and transferrin were obtained by exhaustive pronase digestions of glycoproteins, followed by separation on Sephadex G-25 gel by the procedure of Huang et al. (8). Asparaginyl oligosaccharides of ovalbumin (GP-I to GP-V) were prepared from ovalbumin glycopeptides as described by Huang et al. (8) by using Dowex 50W-X2 column chromatography. Dns derivatives of asparaginyl glycopeptides were prepared by the method of Gray (6), followed by paper chromatography to remove dns sulfonic acid. Dns-GP-IIA, -GP-IIB, -GP-IIIA, -GP-IIIB, and -GP-IIIC were separated by high-performance liquid chromatography by using a reversed-phase column (Inertsil ODS, 4.6 by 250 mm; Gasukuro Kogyo, Japan) by the method of Iwase et al. (10). The structures of the heterogeneous ovalbumin glycopeptides obtained were reported by Kobata and co-workers (29, 30, 37) to be as follows: GP-V, $(Man)_5(GlcNAc)_2$-Asn; GP-IV, $(Man)_6(GlcNAc)_2$-Asn; GP-IIIA, $(GlcNAc)_2(Man)_5(GlcNAc)_2$-Asn; GP-IIIB, $(Man)_7(GlcNAc)_2$-Asn; GP-IIIC, $(GlcNAc)_3(Man)_4(GlcNAc)_2$-Asn; GP-IIA, $(Gal)(GlcNAc)_3$-$(Man)_4(GlcNAc)_2$-Asn; GP-IIB,

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$(GlcNAc)_3(Man)_5(GlcNAc)_2$-Asn; and GP-I, $(Gal)(GlcNAc)_3(Man)_5(GlcNAc)_2$-Asn; Man represents mannose, and Gal represents galactose.

Enzyme assays. Endo-β-GlcNAc-ase activity was assayed with $(Man)_6(GlcNAc)_2$ Asn-Dns (Dns-GP-IV) as the substrate. The reaction mixture was composed of 50 nmol of $(Man)_6(GlcNAc)_2$-Asn-Dns, 1 μmol of sodium acetate buffer (pH 6.0), and the enzyme solution in a total volume of 35 μl. After incubation for an appropriate time at 37°C, the reaction was terminated by the addition of 5 μl of 10% trichloroacetic acid. A portion of the reaction mixture was directly analyzed by high-performance liquid chromatography (Hitachi model L6200 chromatograph equipped with an F-1000 fluorescence spectrophotometer) by using a reversed-phase column (11) (Merck RiChrospher 100 RP-18, 4.6 by 250 mm). The column was eluted with 9% acetonitrile in 25 mM sodium borate buffer, pH 7.5, at 1 ml/min. The product, GlcNAc-Asn-Dns, which was separated from $(Man)_6(GlcNAc)_2$-Asn-Dns, was determined by fluorescence (excitation, 313 nm; emission, 540 nm). One unit was defined as the amount of enzyme yielding 1 μmol of GlcNAc-Asn-Dns per min at 37°C under the assay conditions.

Exoglycosidases were assayed with the appropriate *p*-nitrophenyl glycosides. One unit was defined as the amount yielding 1 μmol of *p*-nitrophenol per min at 37°C. Protease activity was assayed by the method of Krauspe and Scheer (14), with azocasein as the substrate. α-Mannanase activity was determined by using yeast mannan as the substrate, and liberated reducing sugar was measured by the Nelson-Somogyi procedure (23, 25).

Analytical methods. Protein was determined by the method of Lowry et al. (18), with crystalline ovalbumin as the standard. The protein content via column chromatography was determined by measuring absorbance at 280 nm. Neutral sugars were assayed by means of the phenol-H_2SO_4 reaction (4).

Gel electrophoresis. Polyacrylamide slab gel electrophoresis was carried out by the method of Davis (3) by using a 7.5% polyacrylamide gel with Tris-glycine buffer, pH 8.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% acrylamide and 0.1% sodium dodecyl sulfate, with a discontinuous Tris-glycine buffer system, by the method of Laemmli (15). Protein was stained with Coomassie Brilliant Blue R-250.

Molecular weight determination. The molecular weight of the enzyme was determined by gel filtration on Sephadex G-100 (1.8 by 113 cm) by the method of Andrews (1). Elution was carried out with 10 mM potassium phosphate buffer (pH 7.0). The following proteins were used as molecular weight markers: chymotrypsinogen (molecular weight, 25,000), ovalbumin (43,000), bovine serum albumin (67,000), and aldolase (158,000).

Purification of endo-β-GlcNAc-ase. The purification of the endo-β-GlcNAc-ase from the culture fluid of *A. protophormiae* is described below. All procedures in the enzyme purification were carried out at 5°C unless otherwise noted.

(i) Ammonium sulfate precipitation. Solid ammonium sulfate was added to 90% saturation to the clarified culture fluid (1,600 ml) obtained after 72 h of cultivation. After standing overnight, the precipitate was collected by centrifugation (10,000 × *g* for 30 min at 4°C) and dissolved in 10 mM potassium phosphate buffer (pH 7.0). This solution was dialyzed against the same buffer overnight.

(ii) First DEAE-Toyopearl 650M column chromatography. The dialyzed solution was applied to a column (3.5 by 20 cm) of DEAE-Toyopearl 650M equilibrated with 10 mM phosphate buffer (pH 7.0). The column was washed with the same buffer (500 ml) and then with buffer containing 0.2 M NaCl (300 ml), and the enzyme was eluted with buffer supplemented with 0.3 M NaCl. The active fractions (100 ml) were combined and dialyzed against 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl.

(iii) Second DEAE-Toyopearl 650M column chromatography. The dialyzed solution was applied to a column (1.0 by 5.0 cm) of DEAE-Toyopearl equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. The column was washed with the same buffer (50 ml) and then eluted with a linear gradient of 0.1 to 0.3 M NaCl in the same buffer (300 ml). The active fractions (120 ml) were combined and dialyzed against 10 mM phosphate buffer (pH 7.0) including 1.0 M ammonium sulfate overnight with two changes of the buffer.

(iv) Phenyl-Sepharose CL-4B column chromatography. The enzyme solution was applied to a Phenyl-Sepharose CL-4B column (1.0 by 30 cm) equilibrated with 1.0 M ammonium sulfate in 10 mM phosphate buffer (pH 7.0). The enzyme was eluted with a linear gradient formed between 100 ml of 1.0 M ammonium sulfate in 10 mM phosphate buffer (pH 7.0) and 100 ml of 40% ethylene glycol in 10 mM phosphate buffer (pH 7.0). The active fractions (40 ml) were combined and concentrated by pressure dialysis by using a collodion bag (Sartorius).

(v) Sephadex G-100 column chromatography. The concentrated enzyme solution was applied to a Sephadex G-100 column (1.6 by 104 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.0). The enzyme was eluted with the same buffer in 3-ml fractions. The active fractions (33 ml) were combined and concentrated by using a collodion bag. The resulting enzyme preparation was stored at −20°C.

RESULTS

Effect of ovalbumin on endo-β-GlcNAc-ase production. Enzyme production in the supernatant of *A. protophormiae* culture broth at different stages of growth was investigated when the bacterium was grown in medium with or without 0.3% ovalbumin. As shown in Fig. 1, both culture fluids showed similar profiles for pH and cell growth. However, endo-β-GlcNAc-ase activity in the culture fluid with 0.3% ovalbumin was more than 10 times higher than that without ovalbumin. Maximum enzyme activity was observed after 2 to 4 days. On the other hand, as shown in Table 1, all other glycosidase activities were weak, except for β-galactosidase, after 3 days of cultivation and did not markedly increase after the addition of ovalbumin to the medium.

For further examination of endo-β-GlcNAc-ase production, the effect of the medium ovalbumin concentration on enzyme production was investigated after 3 days of cultivation. As shown in Fig. 2, the enzyme activity increased linearly with increasing ovalbumin added to the medium. However, additions greater than 2% ovalbumin to the medium were not so effective.

Effect of various compounds on enzyme production. The bacterium was cultivated in media containing various compounds, and the enzyme activity in each culture fluid was investigated after 72 h of cultivation. As shown in Table 2, besides the addition of ovalbumin, the enzyme was induced with the addition of glycoproteins, such as ribonuclease B and yeast invertase, to the medium. These glycoproteins have high-mannose type oligosaccharides (16, 17). We found that GP-III, -IV, and -V derived from ovalbumin remarkably induced enzyme production. These glycopeptides also have

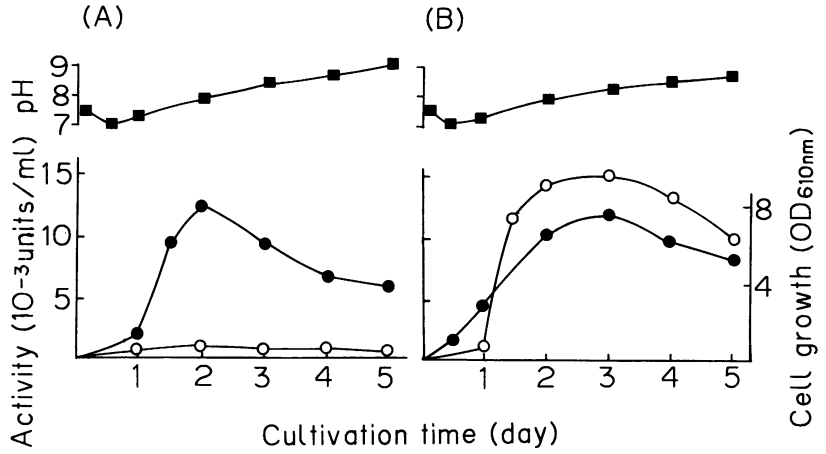

FIG. 1. Growth and enzyme production in cultures of *A. protophormiae*. The cultures were carried out in 500-ml Erlenmeyer flasks without (A) or with (B) 0.3% ovalbumin at 28°C. Symbols: ●, growth; ○, endo-β-GlcNAc-ase; and ■, pH.

high-mannose type oligosaccharides. On the other hand, human transferrin, which has complex type oligosaccharides (26), and GP-I and -II, which have hybrid type oligosaccharides, hardly induced enzyme production. Moreover, monosaccharides such as mannose, galactose, and *N*-acetylglucosamine did not induce the enzyme either.

Enzyme purification. The purification of endo-β-GlcNAc-ase from *A. protophormiae* grown in 1% ovalbumin medium is summarized in Table 3. The enzyme was purified with an overall recovery of about 66%.

Enzyme purity and molecular weight. The purified endo-β-GlcNAc-ase preparation showed a single protein band on polyacrylamide gel and sodium dodecyl sulfate-polyacrylamide gel electrophoreses. The molecular weight of the enzyme was estimated to be 81,000 ± 2,000 by gel filtration. Upon sodium dodecyl sulfate gel electrophoresis, the enzyme exhibited a single band corresponding to a molecular weight of 80,000 ± 1,600. These results indicate that the enzyme has a monomeric structure. The final enzyme preparation was free of other exoglycosidase, chitinase, and protease activities tested.

Effects of pH and temperature. The activity of the purified enzyme at different pHs was examined under the standard assay conditions. All assays were carried out with 100 mM buffers. The enzyme exhibited a very broad optimum pH in the range of pH 5.0 to 11.0 (Fig. 3A). Even at pH 12.0, the enzyme showed about 90% of the maximum activity. The effect of pH on the stability of the enzyme was investigated by storing the enzyme in 50 mM buffers of various pHs at 5°C for 48 h. The stable pH range of the enzyme was from 5.0 to 7.0, and it was also fairly stable at alkaline pHs (Fig. 3B).

The purified enzyme was stable up to 60°C after incubation for 10 min in 100 mM acetate buffer (pH 6.0). The optimum temperature for the enzyme reaction was about 60°C under the assay conditions.

Effects of various compounds. The effects of various metal ions and sulfhydryl reagents on the enzyme activity were examined by preincubating the enzyme with the compound at 37°C for 10 min and assaying the residual activity. The activity of the enzyme was not significantly affected by the following compounds at 1 mM: $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$, $CoCl_2$, $CuSO_4$, EDTA, glutathione, L-cysteine, 2-mercaptoethanol, *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, iodoacetic acid, and *o*-phenanthroline. However, $HgCl_2$ (24% of the original activity), $FeSO_4$ (44%), and $Cd(NO_3)_2$ (54%) inactivated the enzyme.

Substrate specificity. The susceptibility of each asparaginyl

TABLE 1. Various glycosidase activities in two cultures of *A. protophormiae*[a]

Substrates	Enzyme activity (10^{-3} U/ml)	
	−Ovalbumin	+Ovalbumin
p-Nitrophenyl:		
α-Glucose	1.35	1.10
β-Glucose	1.56	0.88
α-Gal	0.97	0.50
β-Gal	4.80	7.10
α-Man	0.49	0.39
β-Man	0.09	0.10
α-Fucose	0.15	0.10
β-Fucose	0.12	0.11
β-Xylose	0.87	0.34
β-GlcNAc	0.17	0.10
Yeast mannan	0	0

[a] *A. protophormiae* was grown in media containing 0.5% peptone, 0.5% yeast extract, and 0.5% NaCl, with or without 1.0% ovalbumin, for 72 h.

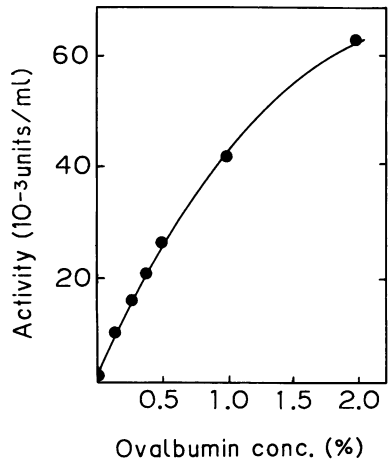

FIG. 2. Effects of ovalbumin concentration on enzyme production in a culture of *A. protophormiae*. Each amount of ovalbumin was added to medium containing 0.5% yeast extract, 0.5% peptone, and 0.5% NaCl. The culture was carried out for 72 h.

TABLE 2. Effects of various compounds on enzyme production by *A. protophormiae*[a]

Addition to medium	Concn (%)	Enzyme activity (%)
Ovalbumin	1.0	100
Ovalbumin-GP-:		
I	0.2	11
II	0.2	12
III	0.2	156
IV	0.2	210
V	0.2	210
Bovine serum albumin	1.0	4
Yeast invertase	1.0	39
Bovine ribonuclease B	1.0	25
Yeast mannan	1.0	9
Human transferrin	1.0	6
Chitotriose	0.2	6
None		4

[a] *A. protophormiae* was grown in media containing 0.5% peptone, 0.5% yeast extact, 0.5% NaCl, and the added compound at the concentration indicated. After 72 h of cultivation, endo-β-GlcNAc-ase activity in the culture fluid was determined relative to that in 1.0% ovalbumin, which was set at 100%. Other sugars such as glucose, galactose, mannose, *N*-acetylglucosamine, glucosamine, and chitobiose had little or no effect on enzyme production.

oligosaccharide of ovalbumin to purified endo-β-GlcNAc-ase was examined by using dansylated derivatives as substrates. About 7 nmol of each of Dns-GP-I to -V was incubated at 37°C with 0.64 mU of the enzyme in 50 mM acetate buffer, pH 6.0 (total volume, 35 μl). The time courses for hydrolysis are shown in Fig. 4A. Under these conditions, Dns-GP-IV and -V were completely hydrolyzed into Dns-Asn-GlcNAc and oligosaccharides, and about 70% of the Dns-GP-III mixture was also hydrolyzed after 6 h of incubation. On the other hand, the enzyme did not hydrolyze Dns-GP-I or -II. Then, for further examination of the hydrolysis of the Dns-GP-III mixture, about 7 nmol of each of Dns-GP-IIIA, -IIIB, and -IIIC, which were separated by high-performance liquid chromatography, was incubated with the enzyme at 37°C. As shown in Fig. 4B, the enzyme completely hydrolyzed Dns-GP-IIIB and -IIIA after 6 h of incubation. However, the enzyme did not hydrolyze Dns-GP-IIIC. At a high concentration of the enzyme (64 mU), 96% of Dns-GP-III and 48% of Dns-GP-I and -II were hydrolyzed after 6 h of incubation. This enzyme did not act on $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$-Asn-Dns complex type oligosaccharide derived from human transferrin.

DISCUSSION

Glycoproteins having asparagine-linked sugar chains are ubiquitous in nature and are found in living organisms, with

TABLE 3. Purification of endo-β-*N*-acetylglucosaminidase from *A. protophormiae*

Source of enzyme or procedure for purification	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)
Culture fluid	3,498[a]	91.6	(0.026)	100
Ammonium sulfate	176	89.3	0.51	97
1st DEAE-Toyopearl	14.5	78.0	5.4	85
2nd DEAE-Toyopearl	11.0	67.0	6.1	73
Phenyl-Sepharose CL-4B	6.1	63.5	10.4	69
Sephadex G-100	1.8	60.0	33.3	66

[a] Undigested ovalbumin was contained in the culture fluid.

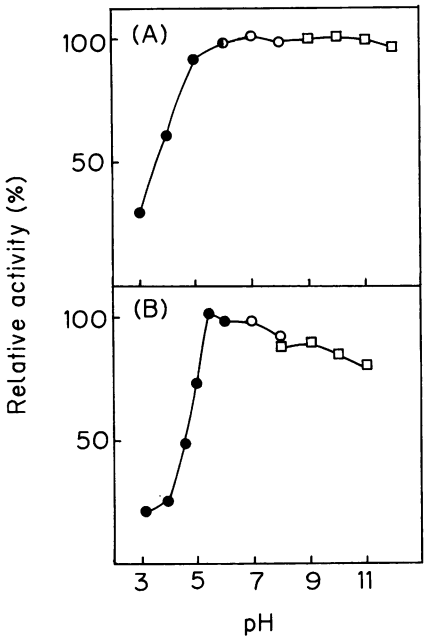

FIG. 3. Effects of pH on the activity and stability of the enzyme. (A) Enzyme assayed in 50 mM buffers of various pHs under standard conditions. (B) Enzyme stored at 5°C for 48 h in various 50 mM buffers, followed by assay of remaining activity. Buffers: ●, sodium acetate-acetic acid; ○, potassium phosphate; □, glycine-NaOH.

the exception of bacteria. Although most bacteria do not produce glycoproteins (20), some of them are good sources of endo-β-GlcNAc-ase. Therefore, we examined for the substrates of this bacterial enzyme. There are many reports

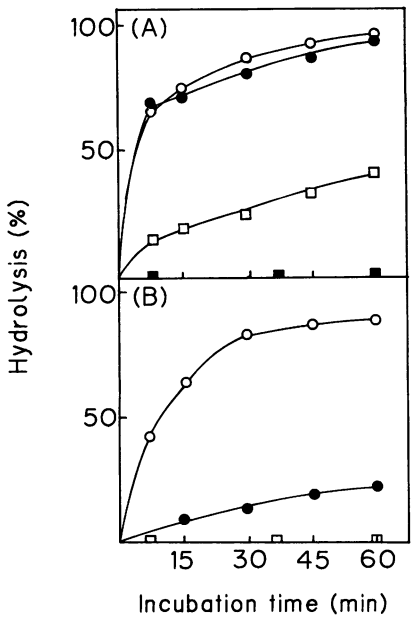

FIG. 4. Hydrolysis of dansylated ovalbumin glycopeptides by the enzyme. The rates of hydrolysis by the enzyme were analyzed as described in the text. Symbols: (A) ○, GP-V; ●, GP-IV; □, GP-III mixture; and ■, GP-II mixture; (B) ○, GP-IIIB; ●, GP-IIIA; and □, GP-IIIC. In panel A, GP-I gave the same results as the GP-II mixture.

concerning the induction of glycosidases that act on polysaccharides such as starch, cellulose, mannan, glucan, and chitin (19, 33). But there are no reports of the induction of endoglycosidases that act on asparagine-linked oligosaccharides of glycoproteins.

When *A. protophormiae* was grown in medium containing ovalbumin, which is a typical glycoprotein having asparagine-linked oligosaccharides, we found that ovalbumin oligosaccharides accumulated in the culture fluid. This fact showed that this bacterium produced endo-β-GlcNAc-ase when grown in medium containing ovalbumin. As described in the text, *A. protophormiae* does not produce α-mannosidase or α-mannanase at all in culture fluid. Therefore, this bacterium does not completely degrade asparagine-linked oligosaccharides. This result suggests that the production of endo-β-GlcNAc-ase is not dependent upon carbon sources. As shown in Fig. 1, the pH of the culture fluid was approximately 9.0, and the endo-β-GlcNAc-ase of *A. protophormiae*, unlike enzymes from other sources, showed high activity at alkaline pHs. We also found that this bacterium produced alkaline protease with a high activity in culture fluid (manuscript in preparation). We already reported that microbial glycoproteins such as yeast invertase and *Rhizopus* glucoamylase become sensitive to proteinases after the deglycosylation of asparagine-linked oligosaccharides by endo-β-GlcNAc-ase (31, 35, 36). Therefore, concerning the action of endo-β-GlcNAc-ase, this alkaline protease of *A. protophormiae* may play an important role in the degradation of glycoproteins.

The *A. protophormiae* endo-β-GlcNAc-ase may specifically act on high-mannose type oligosaccharides. The substrate specificity of the *A. protophormiae* enzyme was similar to that of Endo-C_{II} purified from *Clostridium perfringens* (29). Most endo-β-GlcNAc-ases from microorganisms are highly specific for high-mannose type oligosaccharides. Also, all microbial glycoproteins reported to date have high-mannose type oligosaccharides (7, 9, 22, 32). Therefore, it is reasonable that these enzymes show substrate specificities toward the degradation of microbial glycoproteins. On the contrary, endo-β-GlcNAc-ases from pathogenic bacteria, such as *Diplococcus pneumoniae*, *Flavobacterium meningosepticum*, and *C. perfringens* (C_I enzyme), can act on the core structure or native form of complex type oligosaccharides. Such oligosaccharides are widely distributed in the animal kingdom. Thus, the substrate specificities of the enzymes seem to be influenced by the structures of oligosaccharides encountered in different environments. *A. protophormiae* induced the enzyme when grown in a medium containing glycoproteins which the bacterium was able to degrade. The induction mechanism of the enzyme is currently being investigated. Further studies on the action of *A. protophormiae* endo-β-GlcNAc-ase toward various glycoproteins are in progress.

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