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Purification and Properties of β-*N*-Acetylhexosaminidase from *Mucor fragilis* Grown in Bovine Blood

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***Mucor fragilis* grown on bovine blood powder as the sole carbon source abundantly produced β-*N*-acetylhexosaminidase. The enzyme activity was several times higher than that of a culture obtained with glucose medium. The enzyme had two different molecular weight forms. The high-molecular-weight form had somewhat higher β-*N*-acetylgalactosaminidase activity than the lower-molecular-weight enzyme which had β-*N*-acetylgalactosaminidase activity equivalent to about 40% of its β-*N*-acetylglucosaminidase activity. Bovine blood seemed to induce both enzymes, but *N*-acetylamino sugars specifically induced the low-molecular-weight form. *N*-Acetylgalactosamine had an especially marked effect on activity. The low-molecular-weight form of enzyme was purified from the culture filtrate by fractionation with ammonium sulfate and various column chromatographies. The purified enzyme was found to be homogeneous by polyacrylamide gel electrophoresis. The optimum pH was 4.0 to 5.0 for β-*N*-acetylglucosaminidase activity and 5.5 to 6.5 for β-*N*-acetylgalactosaminidase activity. The enzyme hydrolyzed natural substrates such as di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, and a glycopeptide obtained by modification of fetuin.**

Animal blood produced by inspected slaughterhouses is wasted biomass in many countries. This unusable biomass causes an increase in the biochemical oxygen demand value in drainage. However, its profitable use could help decrease the pollution problems encountered in disposing of blood. In several European countries, blood is used as an ingredient of various foods, such as sausages, puddings, and bread (21).

We have investigated ways of reusing this biomass by using microorganisms. Blood is rich in glycoproteins and glycolipids. We therefore attempted to obtain useful glycosidases produced by microorganisms which can grow on bovine blood as a medium. In the course of the study, we found that some fungi grown on bovine blood as the sole carbon source abundantly produced β-*N*-acetylhexosaminidase. The enzyme activity of *Mucor fragilis* was several times higher than that of a culture grown in glucose medium.

β-*N*-Acetylhexosaminidase (EC 3.2.1.52) is widely distributed among mammalian tissues, higher plants, and microorganisms. The enzyme catalyzes the hydrolysis of the β-*N*-acetylglucosaminyl or β-*N*-acetylgalactosaminyl moiety of the nonreducing end of oligosaccharides and of the sugar chains of glycoconjugates. The enzymes of some fungi, including *Aspergillus niger* (2, 7), *Aspergillus oryzae* (10), *Sclerotinia fructigena* (15), *Tremella fuciformis* (17), *Paecilomyces persicinus* (5), and *Pycnoporus cinnabarinus* (12), were purified from cells or culture filtrates. We also purified an uncommon enzyme from a culture filtrate of *Penicillium oxalicum* which had higher β-*N*-acetylgalactosaminidase (β-GalNAcase) activity than β-*N*-acetylglucosaminidase (β-GlcNAcase) activity (22). Recently, β-*N*-acetylhexosaminidases have been recognized as a very useful tool for elucidation of the structures and functions of glycolipids and glycoproteins, since β-linked *N*-acetylhexosaminyl units are common constituents of biologically important materials such as blood group substances, immunoglobulins, cell walls of microorganisms, etc.

This paper describes the production of an extracellular β-*N*-acetylhexosaminidase by *M. fragilis* grown on bovine blood, the occurrence of enzymes in two different molecular weight (MW) forms, and the purification of the lower-MW form of enzyme.

MATERIALS AND METHODS

Chemicals. Bovine blood lyophilized powder was obtained from Difco Co. Ltd. *p*-Nitrophenyl-β-*N*-acetylglucosaminide (*p*NPGlcNAc) and *p*-nitrophenyl-β-*N*-acetylgalactosaminide (*p*NPGalNAc) were purchased from Nakarai Chemicals Ltd. DEAE-Sepharose CL-6B, Sephadex G-200 and G-150, and concanavalin A-Sepharose 4B were from Pharmacia Fine Chemicals, Inc. Hydroxylapatite was prepared by the method of Tiselius et al. (18). Di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose were prepared from chitin by the method of Rupley (16). Fetuin (from fetal calf serum), neuraminidase (from *Clostridium perfringens*), and β-galactosidase (from bovine liver) were purchased from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Microorganisms and cultivation. Fungi from our collection, including *M. fragilis* IFO6449, were used throughout this study. The medium was composed of 1% bovine blood lyophilized powder, 0.5% peptone, 0.5% yeast extract, and 0.5% NaCl, pH 6.5. A loopful of cells was inoculated into 5 ml of the medium in a test tube, and the seed culture was incubated at 28°C for 2 days with shaking at 250 rpm. One tube of the seed culture was transferred to a 500-ml shake flask containing 120 ml of medium, and cultivation was continued at 28°C on a reciprocal shaker operating at 120 rpm. After cultivation, the culture fluid was filtered through filter paper under reduced pressure, and the clarified supernatant was used as the source of the enzyme.

Enzyme assay. β-GlcNAcase and β-GalNAcase activities were assayed with *p*NPGlcNAc and *p*NPGalNAc, respectively, as substrate. The enzyme solution (10 to 100 μl) was

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added to 0.25 ml of a solution of 2 mM substrate dissolved in 50 mM sodium citrate buffer, pH 4.5. After incubation for an appropriate time at 37°C, 1.75 ml of 0.2 M sodium borate buffer, pH 9.8, was added to terminate the reaction, and the *p*-nitrophenol liberated was determined from absorbance at 400 nm. For these conditions, an extinction coefficient of 1.77×10^4 M^{-1} cm^{-1} was used to calculate the concentration of *p*-nitrophenol in the assay mixture. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of *p*-nitrophenol per min.

Gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed by the method of Davis (3) with a 7.5% polyacrylamide gel and Tris-glycine buffer, pH 8.3. Electrophoresis was carried out at a current of 2 mA per column, and gels were stained for protein with amido black. For localization of the enzyme activity, gels were incubated at 37°C with a mixture of 0.015% naphthol ASBI (6-bromo-2-hydroxy-3-naphthol-*o*-anisidine)-*N*-acetyl-β-D-glucosaminide and 0.01% Fast Garnet GBC (*o*-amino-azotoluene diazonium salt) (13). The part containing the enzyme activity was stained a brownish color. For detection of sugar chains of the enzyme, gels were stained with periodate-fuchsin reagent (23).

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (20). Protein samples were denatured at 37°C for 5 h in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% SDS and 0.1% 2-mercaptoethanol. The samples were applied to a 10% polyacrylamide gel containing 0.1% SDS, and electrophoresis was performed at a current of 8 mA per gel.

MW determination. The MW of the purified enzyme was estimated by gel filtration on a column of Sephadex G-200 by the method of Andrews (1). The column was calibrated with a standard sample containing catalase (MW 232,000), aldolase (MW 158,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), and chymotrypsinogen A (MW 25,000).

Analyses. *N*-Acetylhexosamines were determined by the method of Reissig et al. (14). Protein was determined by the method of Lowry et al. (9) with crystalline egg albumin as the standard. The protein content after column chromatography was determined by measuring the absorbance at 280 nm.

Purification of the enzyme. A typical purification of the low-MW β-*N*-acetylhexosaminidase from the culture filtrate after 5 days of cultivation is described below. All operations in the enzyme purification were carried out at about 5°C.

(i) Concentration with ammonium sulfate. To the clarified culture filtrate obtained after 5 days of cultivation, solid ammonium sulfate was added to 75% saturation, with stirring and adjustment to pH 7.0. After standing overnight, the resulting precipitate was collected by centrifugation at 10,000 × *g* for 30 min and dissolved in 0.01 M potassium phosphate buffer (pH 7.0). The solution was dialyzed overnight against the same buffer. Traces of denatured protein were removed by centrifugation at 10,000 × *g* for 30 min.

(ii) DEAE-Sepharose CL-6B column chromatography. The supernatant was applied to a column (4 by 43 cm) of DEAE-Sepharose CL-6B equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). The column was washed with the same buffer and then eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The first of the two peaks of enzyme activity was pooled and brought to 75% saturation with ammonium sulfate and then left overnight. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min and then dissolved in a small volume of 1 mM potassium phosphate buffer (pH 7.0). The solution was dialyzed against the same buffer.

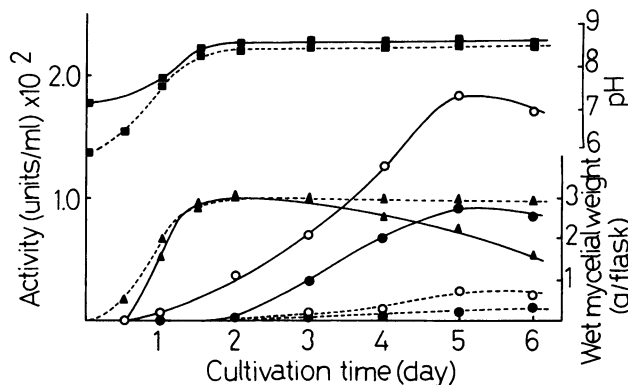

FIG. 1. Growth and enzyme production in cultures of *M. fragilis*. The cultures were carried out in 500-ml shake flasks containing 120 ml of 1% glucose medium (- - - -) or 1% bovine blood medium (——) at 28°C on a reciprocal shaker. Mycelia wet weights were measured after culture fluids were filtered through filter paper. Symbols: ▲, mycelia; ○, β-GlcNAcase; ●, β-GalNAcase; ■, pH.

(iii) Hydroxylapatite column chromatography. The dialyzed solution was applied to a column of hydroxylapatite (1.7 by 23 cm) equilibrated with 1 mM potassium phosphate buffer (pH 7.0). The column was washed with the same buffer before elution with a linear gradient of 1 mM to 0.5 M potassium phosphate buffer (pH 7.0). Fractions containing enzyme activity were pooled and then concentrated by ultrafiltration with a membrane (PM-10; Amicon).

(iv) Sephadex G-150 gel filtration. The concentrated enzyme solution was applied to a Sephadex G-150 column (1.7 by 112 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) and eluted with the same buffer. The active fractions were pooled and concentrated by ultrafiltration.

(v) Concanavalin A-Sepharose 4B column chromatography. The concentrated enzyme solution was applied to a column (1.4 by 7.5 cm) of concanavalin A-Sepharose 4B equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. After the column had been washed with the same buffer, the enzyme was eluted with the above buffer containing 0.5 M α-methylmannoside. Fractions containing enzyme activity were pooled and concentrated by ultrafiltration. The resulting enzyme preparation was stored at −20°C.

RESULTS

Production of β-*N*-acetylhexosaminidase. When *M. fragilis* was grown on bovine blood as the sole carbon source, the β-*N*-acetylhexosaminidase activity in the culture fluid was several times higher than that in the supernatant of a culture grown in 1% glucose medium. The maximum enzyme activity was observed after 4 to 5 days, when fungal autolysis took place. On the other hand, little activity was found when *M. fragilis* was grown on glucose medium, although both the maximum mycelial yield and final pH were almost the same as for cells grown in bovine blood medium (Fig. 1). No glycosidase activities except β-*N*-acetylhexosaminidase were found in the culture fluid of either glucose-grown or bovine blood-grown fungi. The enzyme activity depended on the initial pH of the bovine blood medium; the maximum activity was obtained with an initial pH of about 8.

Two different MW forms of the enzyme. We found two β-*N*-acetylhexosaminidases having different molecular

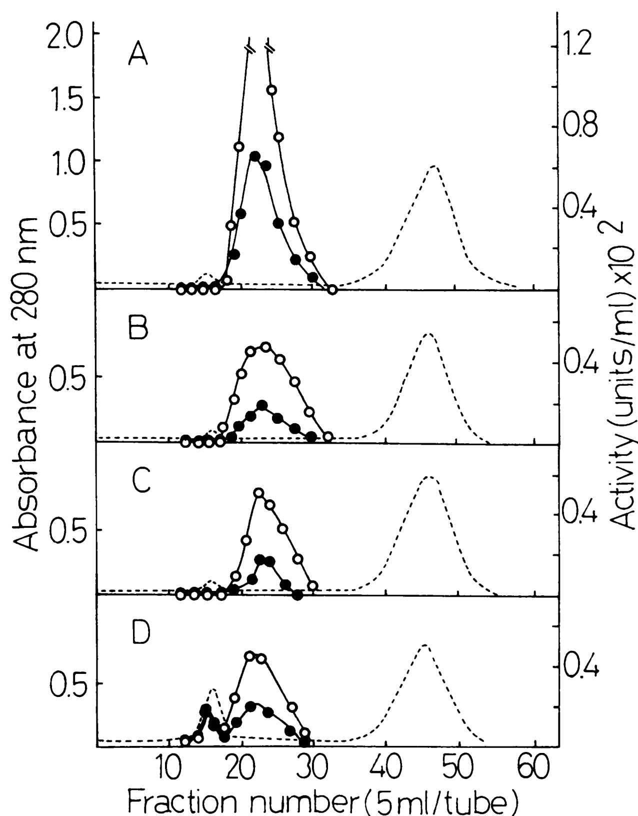

FIG. 2. Sephadex G-150 gel filtration of β-*N*-acetylhexosaminidases produced with various amino sugars. (A) 0.5% GalNAc medium; (B) 0.5% GlcNAc medium; (C) 0.5% *N*-acetylmannosamine medium; (D) 1% bovine blood medium. Fraction volume, 5 ml. Symbols: ○, β-GlcNAcase; ●, β-GalNAcase; - - - -, absorbance at 280 nm.

weights in the culture fluid on Sephadex G-150 gel filtration only when the fungus was cultivated on bovine blood medium. The high-MW form of enzyme (named EI) seemed to be induced by bovine blood because it almost completely disappeared in the culture fluid with glucose medium. It had somewhat higher β-GalNAcase activity than the other enzyme, a low-MW form (named EII), which showed mainly β-GlcNAcase activity (Fig. 2D). The activity of the low-MW form of enzyme was also enhanced by bovine blood.

Effect of various carbon sources on enzyme production. The fungus was cultivated on media in which various carbon sources replaced the glucose, and the enzyme activity in each culture filtrate was investigated. β-*N*-Acetylhexosaminidase increased after the addition of *N*-acetylamino sugars to the medium. GalNAc had an especially marked effect on the activity (about 17-fold higher than in glucose medium). Maximum activity was obtained with 0.8% GalNAc added to the medium. These amino sugars seemed to specifically induce the low-MW form of enzyme (EII) (Fig. 2).

Purification of β-*N*-acetylhexosaminidase. We tried to purify the two enzymes, EI and EII, in the culture filtrate of bovine blood-grown fungi, but EI was produced in smaller amounts and was also rather unstable compared with EII. We did not succeed in purifying EI, but the purification of EII is summarized in Table 2. The overall purification was about 3,500-fold, with a yield of 10% as judged by assay of β-GlcNAcase. At the DEAE-Sepharose CL-6B column chromatography step, most of EI was separated from EII: EII eluted first (0.2 M NaCl), followed by EI (0.4 to 0.5 M NaCl), on stepwise elution with an increasing NaCl concentration. After this step, the ratio of the β-GlcNAcase and β-GalNAcase activities of EII remained almost constant throughout the purification procedure (Table 2). The purified enzyme showed β-GalNAcase activity equivalent to about 40% of the β-GlcNAcase activity.

Purity and MW. The purified β-*N*-acetylhexosaminidase preparation (EII) showed a single protein band on polyacrylamide gel electrophoresis, and the position of the protein band coincided with that of the bands on activity-stained and sugar-stained gels (Fig. 3). This enzyme seemed to be a glycoprotein.

The MW of the enzyme was determined by gel filtration on a Sephadex G-200 column at approximately 125,000. Upon SDS gel electrophoresis, the enzyme exhibited a single band corresponding to an MW of about 70,000. Therefore, the enzyme consists of two subunits of identical MW.

Effect of pH on activity and stability. The activity of the purified enzyme (EII) at different pHs is shown in Fig. 4a. All assays were carried out with various 0.05 M buffers (sodium citrate-HCl, sodium citrate-citric acid, potassium phosphate, Tris hydrochloride, glycine-NaOH). The optimum pH was 4.0 to 5.0 for β-GlcNAcase activity and 5.5 to 6.5 for β-GalNAcase activity. The effect of pH on the stability of the enzyme was investigated by storing the enzyme in 0.05 M buffers of various pHs at 4°C for 46 h. The stable pH range of the enzyme was relatively wide, from pH 4.5 to 8.5 (Fig. 4b). Both activities showed the same extent of impairment at the various pHs.

Thermal stability. The purified EII enzyme was stable up to about 45°C after incubation for 10 min in 0.01 M potassium phosphate buffer (pH 7.0), and 50% of the original activity remained after incubation at 57.5°C for 10 min. The activities of both β-GlcNAcase and β-GalNAcase showed the same dependence on temperature.

Effects of various compounds. The effects of various compounds on the enzyme (EII) activity were investigated. Incubation was for 15 min at 37°C, and then the residual activity was measured under standard conditions. Most of the SH-protective and SH-inhibitory reagents tested (such as dithiothreitol, 2-mercaptoethanol, *p*-chloromercuric benzoate, *N*-ethylmaleimide, etc.) had no significant effect on the enzyme activity. Only Hg^{2+} inhibited both β-GlcNAcase and β-GalNAcase activity at a concentration of 0.25 mM. All sugars (10 mM) and divalent cations (1 mM) tested also had

TABLE 1. Effects of various carbon sources on enzyme activity[a]

Addition to medium	Concn (%)	β-GlcNAcase activity (%)
Bovine blood	1.0	100
Glucose	0.5	18
Galactose	0.5	9
Mannose	0.5	19
Sucrose	0.5	79
Lactose	0.5	82
Glucosamine	0.5	80
Galactosamine	0.5	99
Mannosamine	0.5	94
GlcNAc	0.5	136
GalNAc	0.5	1,730
N-Acetylmannosamine	0.5	128

[a] *M. fragilis* was grown on medium containing 0.5% peptone, 0.5% yeast extract, 0.5% NaCl, and a carbon source at the concentration indicated. After 5 days of culture, the β-*N*-acetylhexosaminidase activity in the culture filtrate was determined relative to that in bovine blood medium, which was set at 100%.

TABLE 2. Purification of β-*N*-acetylhexosaminidase from *M. fragilis*

Step	Total protein (mg)	β-GlcNAcase			β-GalNAcase		Ratio of total U, β-GlcNAcase/β-GalNAcase
		Total U	Sp act (U/mg)	Yield (%)	Total U	Sp act (U/mg)	
Culture filtrate	7,820	78.2	0.010	100	54.7	0.007	0.70
$(NH_4)_2SO_4$ precipitation	1,750	77.0	0.044	98	40.3	0.023	0.52
DEAE-Sepharose CL-6B column chromatography	251	42.7	0.17	55	17.1	0.068	0.40
Hydroxylapatite column chromatography	11.2	22.7	2.03	29	9.7	0.87	0.43
Sephadex G-150 column chromatography	3.1	18.8	6.07	24	7.3	2.35	0.39
Concanavalin A-Sepharose 4B column chromatography	0.22	7.7	35.2	10	3.1	14.2	0.40

little or no effect on enzyme activity. Neither β-GlcNAcase nor β-GalNAcase activity was inhibited by GlcNAc or GalNAc, unlike other mold enzymes (5, 11, 12, 17).

K_m and substrate specificity. The rate of hydrolysis with respect to substrate concentration was examined. The apparent Michaelis constants of EII, calculated from Lineweaver-Burk plots, were 0.28 mM for *p*NPGlcNAc and 0.46 mM for *p*NPGalNAc.

The activities of the enzyme for naturally occurring compounds were investigated with di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, and glycopeptide obtained by exhaustive digestion of fetuin with neuraminidase and β-galactosidase. The EII enzyme hydrolyzed di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose and liberated GlcNAc residues. The hydrolysis rate for di-*N*-acetylchitobiose was faster than that for tri-*N*-acetylchitotriose. Also, the enzyme was found to be capable of hydrolyzing β-galactosidase-treated desialized fetuin. The enzyme liberated GlcNAc from lysozyme-treated cell walls of *Micrococcus lysodeikticus*.

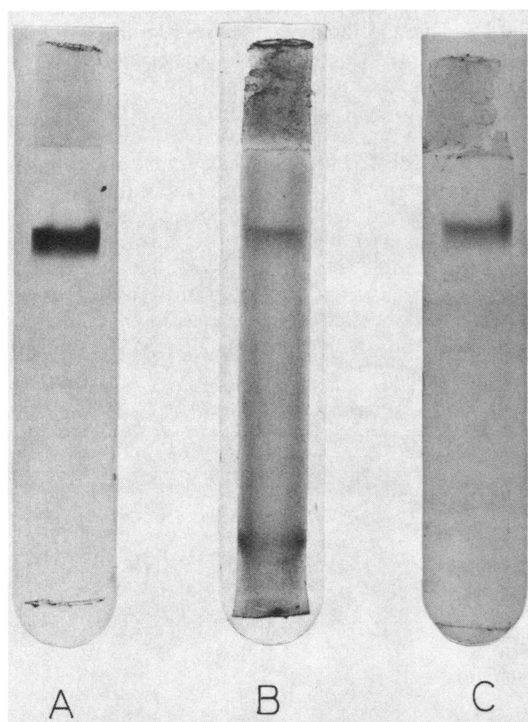

FIG. 3. Polyacrylamide gel electrophoresis of the purified β-*N*-acetylhexosaminidase. The enzyme was applied to 7.5% polyacrylamide gels. After electrophoresis, gels were stained for protein with amido black (A), for the localization of enzyme activity with naphthol ASBI–*N*-acetyl-β-D-glucosaminide and Fast Garnet GBC (B), and for sugar chains with periodate-fuchsin reagent (C).

DISCUSSION

Blood is sometimes referred to as liquid meat because its nutritive value is almost equal to that of lean meat. Recently, some attempts have been made to use animal blood in meat products. It has been expected that blood could be used as a potential source of large quantities of dietary protein (19, 21). In the course of our investigation on the reuse of this biomass, we used bovine blood as a medium for microorganisms and tried to obtain useful enzymes produced by the microorganisms. We found that whole bovine blood is a potent carbon source for cultivating microorganisms, and various glycosidases were produced in the culture fluid when microorganisms were cultivated on bovine blood medium. The β-*N*-acetylhexosaminidase activity of *M. fragilis* was high in comparison with that obtained in glucose medium, suggesting that a component in bovine blood induces the enzyme.

Furthermore, *M. fragilis* β-*N*-acetylhexosaminidases with two different MW forms were found in the culture filtrate of bovine blood medium. This is very rare in microorganisms, because it has been reported that β-*N*-acetylhexosaminidases from microorganisms do not have isozyme forms, except for a *Streptococcus* sp. (8) and *Dictyostelium discoideum* (4), in contrast to the enzymes from animal tissues. The high-MW form of enzyme seemed to be specifically induced by bovine blood, though the activity of the low-MW form of enzyme also increased. Since the high-MW enzyme had rather higher β-GalNAcase activity, it may be able to act on glycolipids, which commonly have β-GalNAc linkages, although no enzyme that can cleave this linkage of glycolipids has been found previously in microorganisms. It was reported that β-GlcNAcase from a *Streptomyces* sp. was induced by chitooligosaccharide or GlcNAc (6). The *M. fragilis* enzyme was also induced by *N*-acetylamino sugars, especially GalNAc. But amino sugars induced only the low-MW form of enzyme, never the high-MW form. *M. fragilis* originally had the lower-MW enzyme because the enzyme was detectable when the fungus was grown on glucose medium. However, it is curious that the activity of the original enzyme was increased more by the addition of GalNAc than by the addition of GlcNAc to the medium.

The properties of the enzyme from *M. fragilis* are not significantly different from those reported for this enzyme from other fungi. However, the enzyme was not inhibited by GlcNAc or GalNAc alone or together; these results are different from those for the β-*N*-acetylhexosaminidases of other fungi reported previously (5, 11, 12, 17). The enzyme

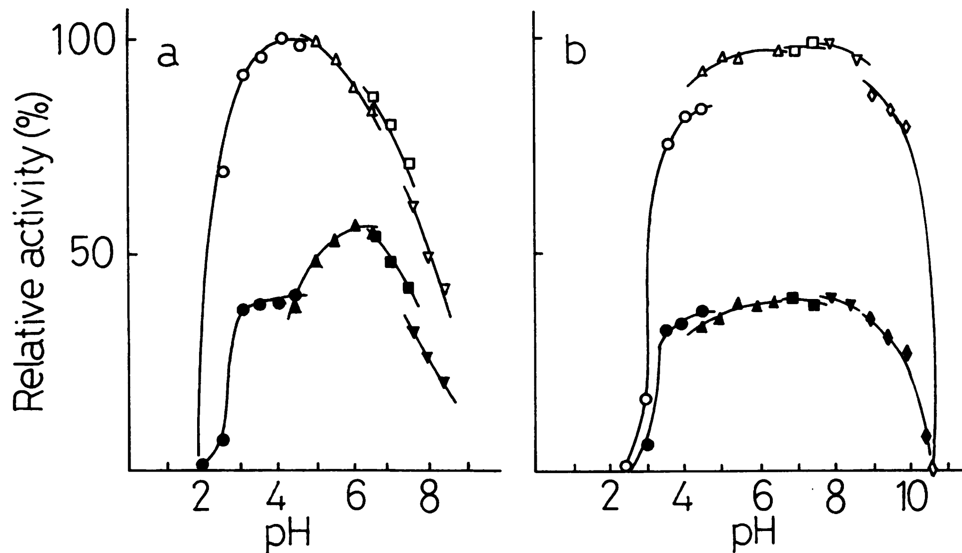

FIG. 4. Effects of pH on activity and stability of β-*N*-acetylhexosaminidase. (a) Enzyme activity was assayed in 0.05 M buffers of various pHs under standard conditions, and β-GalNAcase activity was expressed relative to that of β-GlcNAcase. (b) Enzyme was kept at various pHs at 4°C for 46 h in various 0.05 M buffers, followed by assay of the remaining activity under standard conditions. β-GalNAcase activity was expressed relative to that of β-GalNAcase. Buffers: ○,●, sodium citrate-HCl; △,▲, sodium citrate-citric acid; □,■, potassium phosphate; ▽,▼, Tris hydrochloride; ◇,◆, glycine-NaOH. Open symbols, β-GlcNAcase; solid symbols, β-GalNAcase.

has an advantage as an analytical tool in that it is not inhibited by the reaction products (amino sugars).

In addition to the artificial substrates *p*NPGlcNAc and *p*NPGalNAc, the *M. fragilis* enzyme hydrolyzed the β-*N*-acetylhexosaminyl linkage of oligosaccharides such as di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose, as shown for the enzymes isolated from other fungi (2, 11, 12, 17) and for glycopeptide obtained from fetuin in blood plasma. Thus, the enzyme from *M. fragilis* grown on bovine blood medium may be valuable for structure studies on glycoconjugates which contain β-GlcNAc or β-GalNAc.

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