PURIFICATION AND CHARACTERIZATION OF ß-*N*-ACETYLHEXOSAMINIDASE FROM THE LIVER OF JAPANESE COMMON SQUID *TODARODES PACIFICUS*

Masahiro Matsumiya, Nobuhiro Suzuki, Humiko Tanaka, and Masahiko Shigeo

Department of Marine Science and Resources, College of Bioresource Sciences,
Nihon University, Fujisawa, Kanagawa 252-8510, Japan
E-mail: matsumiya@brs.nihon-u.ac.jp

Abstract

ß-*N*-Acetylhexosaminidase was purified from the liver of Japanese common squid *Todarodes pacificus* by ammonium sulfate fractionation (0-70%) and column chromatographies on Butyl-Toyopearl 650S and Toyopearl HW-55SS. The purified enzyme showed single protein band on PAGE. The molecular weight of the enzyme were estimated to be 125 kDa by gel filtration, 54 kDa by SDS-PAGE in non-reducing condition, and 33 kDa by SDS-PAGE in reducing condition. The optimum pH and temperature were 4.0 and 70 ^{o}C, respectively. The enzyme was stable from pH 3.5 to 5.5, and below 60 ^{o}C, respectively. The K_m value of the ß-*N*-acetylhexosaminidase for *p*-nitrophenyl *N*-acetylglucosaminide (pNp-GlcNAc) was 0.23 mM. As the GlcNAc-chain length of the substrate increases from pNp-GlcNAc to pNp Tri-*N*-acetylchitotorioside (pNp-$GlcNAc_3$), the release of pNp was delayed. The enzyme produced GlcNAc of ß-anomer from $GlcNAc_3$ by enzymatic hydrolysis. These results indicate that ß-*N*-acetylhexosaminidase from the liver of Japanese common squid releases GlcNAc from the non-reducing end side.

Introduction

Chitin is a polysaccharide in which ß-1,4 is linked to *N*-acetyl-D-glucosamine (GlcNAc) and it is known as a major component of exoskeleton of crustacean. Chitinase (EC3.2.1.14) decomposes chitin to *N*-acetylchitooligosaccharides ($GlcNAc_n$), and ß-*N*-acetylhexosaminidase (EC3.2.1.52) further decomposes it to GlcNAc. GlcNAc is a suitable functional material with sweetness for the food industry. Therefore, ß-*N*-acetylhexosaminidase is useful enzyme as an exo-type chitinolytic enzyme for enzymatic production of GlcNAc [1]. We investigated purification and characterization of ß-*N*-acetylhexosaminidase from the liver of Japanese common squids which are waste produced in the process of food processing.

Material and Methods

Materials Fresh Japanese common squid was purchased from Tokyo Wholesale Fish Market. The liver was collected and kept at -80°C until use. $GlcNAc_n$ (n = 1 to 3) and pNp-$GlcNAc_n$ (pNp-$GlcNAc_n$) (n = 1 to 3) were purchased from Seikagaku Kogyo Co.

Measurement of ß-N-acetylhexosaminidase activity Measurement of ß-*N*-acetylhexosaminidase activity was made according to the method prescribed by Ohtakara [2] using pNp-GlcNAc as substrate. Five-tenths milliliter of crude enzyme solution and 0.2 ml of 4 mM pNp-GlcNAc were added to 0.5 ml of 0.2 M phosphate-0.1 M citrate buffer solution (pH 4.5). The mixture was then reacted for 10 min at 37°C. The reaction was stopped by adding 2 ml of 0.2 M sodium carbonate, and then absorbency was measured at 420 nm. The value recorded was converted into an amount of *p*-nitrophenol by the standard curve prepared using *p*-nitrophenol.

Purification of ß-N-acetylhexosaminidase The liver (100 g) was homogenized with five volumes of 10 mM sodium phosphate buffer (pH 6.2) and centrifuged at 10,000 x *g* for 20 min. The supernatant was filtered through two layers of gauze to remove floating fat and used as the crude extract. Ammonium sulfate was added to the crude extract up to 70% saturation and the precipitate was collected by centrifugation at 10,000 x *g* for 30 min. The precipitate was dissolved in a small volume of 20 mM sodium acetate buffer (pH 5.0) and dialyzed against the same buffer. The dialysate was centrifuged at 12,000 x *g* for 30 min to remove insoluble material. Ammonium sulfate was added to the supernatant up to 1M. The enzyme solution was applied to a Butyl-Toyopearl 650S column (1.5 x 20cm) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0) containing 1M ammonium sulfate. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of ammonium sulfate from 1.0 to 0 M in the same buffer. Active fractions were pooled, dialyzed against distilled water, and freeze dried. The freeze dried enzyme was dissolved in a small volume of 20 mM sodium acetate buffer (pH 5.0) containing 0.1M NaCl and applied to a Toyopearl HW-55SS column (2.6 x 65cm) previously equilibrated with same buffer. Enzyme was eluted with the same buffer. Fractions containing chitinase activity were pooled and this gel filtration was repeated.

Protein measurement Protein concentration was measured by the method of Bradford [3] using bovine serum albumin as the standard protein.

Gel Electrophoresis Polyacrylamide gel electrophoresis (PAGE) was done at pH 4.3 on 12.5% polyacrylamide gel by the procedure described by Reisfeld [4]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% polyacrylamide gel according to the manufacturer's instructions (Phast Gel, Amersham Biosciences). The proteins in the gels were stained with Coomassie brilliant blue R-250.

Anomer formation The analysis was made according to the method prescribed by Karasuda *et al*. [5] using $GlcNAc_3$ as the substrate at pH 4.0. A 20-µl portion was analyzed by HPLC using a Tosoh TSK-Gel amide-80 column (0.46 ID x 25cm).

Results and Discussion

Purification of Japanese common squid ß-N-acetylhexosaminidase As shown in Fig. 1, ß-*N*-acetylhexosaminidase in the Japanese common squid liver was fractionated to several active peaks by a Butyl-Toyopearl 650S column chromatography. This result means that the liver has several ß-*N*-acetylhexosaminidase isozymes. Therefore, we purified one of those, indicated by arrow. The finally purified enzyme showed single protein band on PAGE. The enzyme was purified 479-fold with a recovery of 2.1%. The specific activity of the purified enzyme was 100.6 µmol/mg·min. We also measured K_m value by Lineweaver-Burk double reciprocal plots. The K_m value of the ß-*N*-acetylhexosaminidase for pNp-GlcNAc was 0.23 mM. This value was slightly higher than 0.137mM from prawn liver ß-*N*-acetylglucosaminidase [6].

Molecular weight of Japanese common squid ß-N-acetylhexosaminidase The molecular weight of the enzyme were estimated to be 125 kDa by gel filtration. As shown in Fig. 2, the molecular weight of the enzyme were estimated to be 54 kDa by SDS-PAGE in non-reducing condition and 33 kDa by SDS-PAGE in reducing condition. These results mean that Japanese common squid ß-*N*-acetylhexosaminidase is a homo-tetramer with monomeric molecular weight of 33 kDa. But, monomer of 33 kDa was bound by S-S bounding to make dimmer and produced dimmer was bound by non S-S bounding to make tetramer. This property was similar to ß-*N*-acetylglucosaminidase of *Octopus vulgaris* which is tetramer and 12 kDa of molecular weight [7].

Effects of pH and temperature on the activity and stability of Japanese common squid ß-N-acetylhexosaminidase The enzyme activity was measured at 37°C for 10 min incubation at various pH values. As shown in Fig. 3A, the enzyme showed optimum pH at 4.0. This value is slightly lower than pH 5.0 from common octopus liver [8]. The enzyme was preincubated at 37℃ for 30 min at various pHs, the remaining activity was measured. The enzyme was stable from pH 3.5 to

5.5. Regarding the temperature, as shown in Fig. 3B, the enzyme activity was measured at various temperatures for 10 min incubation at pH 4.5. The enzyme showed optimum temperature at 70℃. The enzyme was preincubated at pH 4.5 for 10 min at various temperatures, the remaining activity was measured. The enzyme was stable still 60℃ and completely inactivated at 80℃ for 10 min incubation.

*Cleavage patterns of p-nitrophenyl N-acetylchitooligosaccharides and Tri-*N*-acetylchitotorioside*

p-Nitrophenyl $GlcNAc_n$ and $GlcNAc_3$ were used to identify the cleavage pattern by Japanese common squid ß-*N*-acetylhexosaminidase. As shown in Fig. 4, a large quantity of *p*-nitrophenol was released with time from pNp-GlcNAc, but a little of *p*-nitrophenol was observed in the reaction with pNp-$GlcNAc_2$. Furthermore, as shown in Fig. 5, the enzyme produced GlcNAc of ß-anomer from $GlcNAc_3$ by enzymatic hydrolysis. These results indicate that ß-*N*-acetylhexosaminidase from the liver of Japanese common squid releases GlcNAc from the non-reducing end side.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research of Japan Society for the Promotion of Science and 'Open Research Center Projects' of Ministry of Education, Culture, Sports, Science and Technology of Japan.

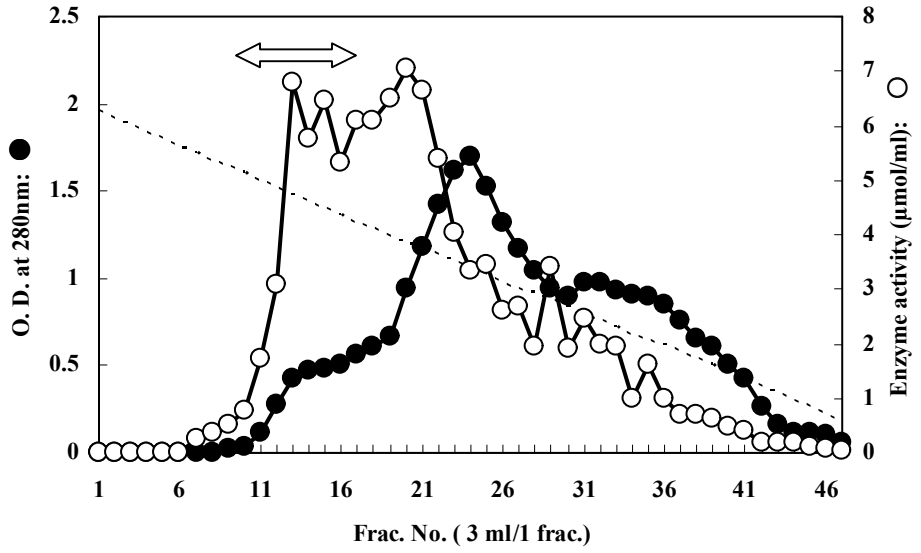

Fig. 1. Butyl-Toyopearl 650S column chromatography of Japanese common squid ß-*N*-acetylhexosaminidase. Buffer: 20 mM sodium acetate buffer (pH 5.0) containing 1M ammonium sulfate. Elution: A linear gradient of ammonium sulfate from 1.0 to 0 M, ----------.

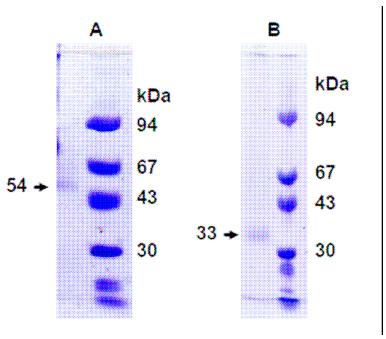

Fig. 2. SDS-PAGE of Japanese common squid ß-*N*-acetylhexosaminidase. A: Non-reducing condition. B: Reducing condition.

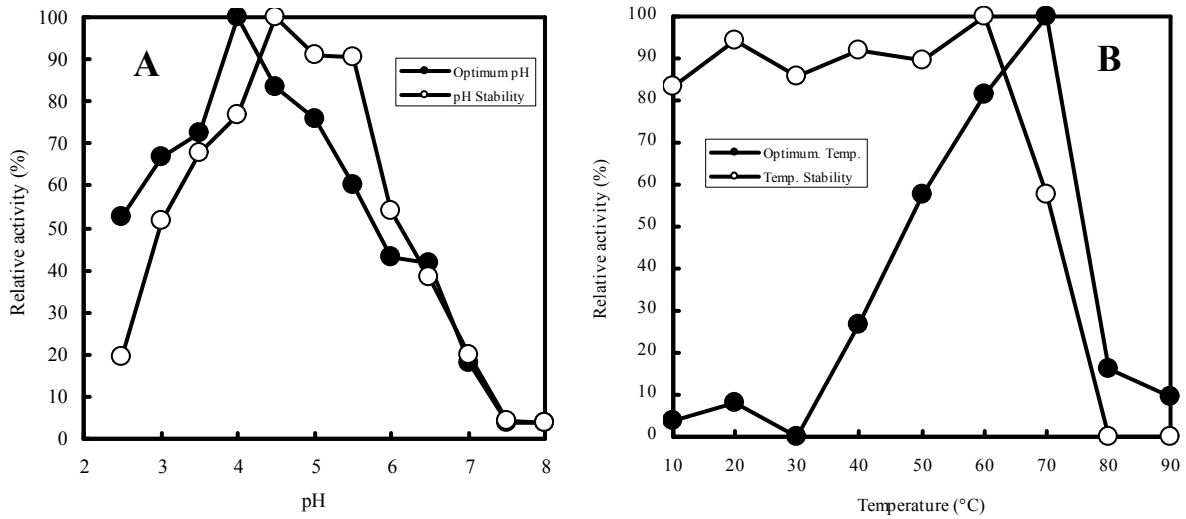

Fig. 3. Effects of pH and temperature on the activity and stability of Japanese common squid ß-*N*-acetylhexosaminidase. A: Effects of pH on the activity and stability of the enzyme. B: Effects of temperature on the activity and stability of the enzyme.

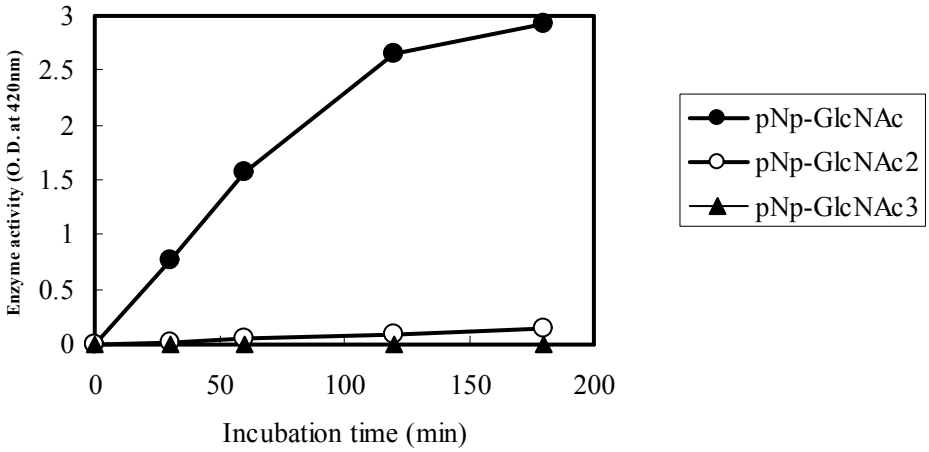

Fig. 4. *p*-Nitrophenyl $GlcNAc_n$ hydrolyzing activity of Japanese common squid ß-*N*-acetylhexosaminidase.

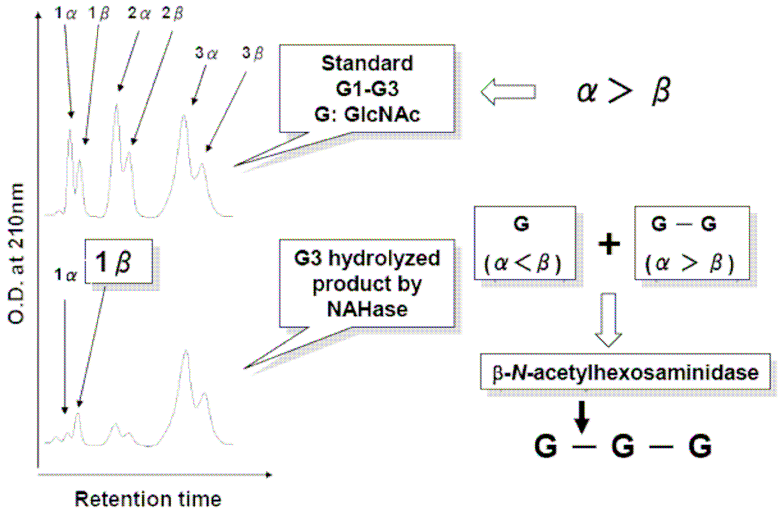

Fig. 5. HPLC analysis of the hydrolysis product of $GlcNAc_3$ by Japanese common squid ß-*N*-acetylhexosaminidase.

References

[1] Matsumiya, M., Enzymatic production of *N*-acetyl-D-glucosamine using crude enzyme from the liver of squids. *Food Sci. Technol. Res*., **10**, 296-299 (2004).

[2] Ohtakara, A., Chitinase and β-*N*-acetylhexosaminidase from *Pycnoporus cinnabarinus*. In "Methods in Enzymology, Vol. 161", eds. Wood, W. A., and Kellogg, S. T., Academic Press, New York, pp. 462-470 (1988).

[3] Bradford, M., A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72**, 248-254 (1976).

[4] Reisfeld, R. A., Lewis, U. J., and Williams. D. E., Disk electrophoresis of Basic proteins and peptides on polyacrylamide gels. *Nature*, **195**, 281-283 (1962).

[5] Karasuda, S., Yamamoto, K., Kono, M., Sakuda, S., and Koga, D., Kinetic analysis of a chitinase from red sea bream, *Pagrus major*. *Biosci. Biotechnol. Biochem*., **68**, 1338-1344 (2004).

[6] Koga, D., Hoshika, H., Matsushita, M., Tanaka, A., Ide, A., and Kono, M., Purification and characterization of β-*N*-acetylhexosaminidase from the liver of a prawn, *Penaeus japonicus*. *Biosci. Biotechnol. Biochem*., **60**, 194-199 (1996).

[7] D'Aniello, A., Cacace, M. G., Atkinson, P. H., and Ceccarini, C., Purification and characterization of β-*N*-acetylglucosaminidase from *Octopus vulgaris*. *Eur. J. Biochem*., **132**, 469-476 (1983).

[8] Nakagawa, H., Enomoto, N., and Asakawa, M., Purification and enzymatic properties of β-*N*-acetylhexosaminidase from common octopus liver. *Nippon Suisan Gakkaishi*, **53**, 1025-1031(1987).