Chemical and Immunological Characterization of the Two α -N-Acetylgalactosaminidases from Squid Liver

Golam SADIK,^a Md Harun or RASHID,^a Tomoko ITOH-NASHIDA,^b Kazutaka ISHII,^a Yoichi SATO,^a Takayuki SHIRAIISHI,^c and Yutaka UDA^{*,a}

^aDepartment of Health Chemistry, Faculty of Pharmaceutical Science, Niigata University of Pharmacy and Applied Life Sciences; Akiha, Niigata, Niigata 956–8603, Japan; ^bDepartment of Biochemistry, School of Life-Dentistry at Niigata, The Nippon Dental University; Chuo, Niigata, Niigata 951–8580, Japan; and ^cLaboratory of Health Chemistry, Nihon Pharmaceutical University; Kita-Adachi-gun, Saitama 362–0806, Japan.

Received February 4, 2009; accepted May 14, 2009; published online June 1, 2009

Based on the inherent α -galactosidase activity, squid liver contains two different α -N-acetylgalactosaminidases (α -GalNAcases): α -N-acetylgalactosaminidase I (α -GalNAcase I), which typically exhibits the α -galactosidase activity and α -N-acetylgalactosaminidase II (α -GalNAcase II), which is devoid of such activity. The molecular properties of the α -GalNAcases that may account for their enzymological differences are as yet unknown. In this study, we have characterized and compared the chemical and immunological properties of α -GalNAcase I and α -GalNAcase II. Analysis of the N-terminal sequence of the first twenty amino acids revealed the striking homology between α -GalNAcase I and α -GalNAcase II. Digestion of α -GalNAcase I and α -GalNAcase II generated the peptide maps that display similarities in peptide pattern, indicating their close relationship in structure. Polyclonal antibodies were generated in rabbits against the purified α -GalNAcase I and α -GalNAcase II for comparison of the immunological properties. Both Western blot and surface plasmon resonance (SPR) studies showed that the anti- α -GalNAcase II antibody reacted with both α -GalNAcase I and α -GalNAcase II, whereas the anti- α -GalNAcase I antibody reacted only with α -GalNAcase I, indicating the presence of common as well as unique antigenic determinants on α -GalNAcase I and α -GalNAcase II. Taken together, these results suggest that α -GalNAcase I and α -GalNAcase II are closely related with regard to structure and that their nonhomologous domains are possibly responsible for the differences in enzymatic properties.

Key words squid liver; α -N-acetylgalactosaminidase; immunological property; N-terminal amino acid sequence; peptide map

α -N-Acetylgalactosaminidase (α -GalNAcase) [EC 3.2.1.49] is a lysosomal exoglycosidase that cleaves α -linked N-acetylgalactosaminyl (α -GalNAc) units from glycoconjugates. Its physiological substrates presumably are glycopeptides and glycoproteins containing O-glycosidic core structures, and oligosaccharides, glycoproteins and glycosphingolipids bearing blood group A determinants or Forssmann haptens.¹⁾ The ability of α -GalNAcase to cleave a wide variety of substrate suggests that this enzyme plays a significant role in the metabolism of glycoconjugates. Mutation in the α -GalNAcase gene identified in patients with Schindler disease²⁾ and Kan-zaki disease³⁾ have been shown to impair the enzymatic activity, resulting in the accumulation of undegraded glycoconjugates in lysosome.^{4,5)} Despite the physiological importance, α -GalNAcase has received much attention recent time for its ability to convert blood group O antigen, the universal donor blood type, from blood group A antigen. α -GalNAcase, therefore, can be used to process human blood to produce type O.^{6,7)} Furthermore, α -GalNAcase is a tool of choice for elucidating the biological functions of the complex glycoconjugates having α -linked N-acetylgalactosaminyl units.

α -GalNAcase, soon after its isolation from human tissue, was thought to be an isoenzyme of α -galactosidase for its weak α -galactosidase activity and thereafter called also α -galactosidase B.^{8,9)} The two enzymes had similar physicochemical properties, a subunit molecular mass of 48 kDa, homodimeric structure and a similar amino acid composition. However, subsequent kinetic, inhibitor, immunologic and gene mapping studies demonstrated that α -galactosidases A and B were genetically distinct lysosomal enzymes with different substrate specificities and α -galactosidase B was in re-

ality an α -GalNAcase.^{10,11)} Thus far, α -GalNAcases purified from different animal tissues including human, bovine, porcine, earthworm, snail, gastropod *Turbo cornutus*, limpet *Patella vulgata* and skipjack, all exhibited the α -galactosidase activity.^{1,12–14)} α -GalNAcases without α -galactosidase activity have been found only in *Clostridium perfringens*.¹⁵⁾

During work on the glycosidases, we first discovered the occurrence of two different α -GalNAcases, α -GalNAcase I and α -GalNAcase II, in the squid liver based on the enzymatic properties.¹⁶⁾ α -GalNAcase I appeared to be a typical α -GalNAcase as it exhibited an inherent α -galactosidase activity and broader substrate specificity.^{16,17)} It was able to hydrolyze the artificial substrates *p*-nitrophenyl- α -N-galactosaminide and *p*-nitrophenyl- α -D-galactoside and natural substrates asialo bovine submaxillary mucin (ABSM), Forssman glycolipid, human ovarian cyst A-glycoprotein, blood group A-type ghosts, ceramide trihexoside (CTH) and oligosaccharides melibiose, raffinose, stachyose, and α -methyl-galactoside, indicating its specificity for α -N-galactosaminyl and α -galactosyl moiety. Moreover, the enzyme was heat stable and its enzymatic activity was inhibited by N-acetylgalactosamine and galactose. Unlike α -GalNAcase I, α -GalNAcase II was devoid of α -galactosidase activity and hydrolyzed only *p*-nitrophenyl- α -N-galactosaminide, ABSM, Forssman glycolipid, human ovarian cyst A-glycoprotein and blood group A-type ghosts, demonstrating its stringent specificity for α -N-galactosaminyl moiety. The enzyme was heat labile and galactose did not affect the enzyme activity. The occurrence of such a novel α -GalNAcase was first described in the animal kingdom. However, the molecular properties of α -GalNAcase I and α -GalNAcase II that may account for

* To whom correspondence should be addressed. e-mail: uda@nupals.ac.jp

their enzymological differences has not yet been examined.

In this study, we made a chemical and immunological comparison between α -GalNAcase I and α -GalNAcase II utilizing N-terminal sequencing, peptide mapping and immunological techniques.

MATERIALS AND METHODS

Materials *p*-Nitrophenyl α -D-*N*-acetylgalactosaminide and tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin were purchased from Sigma Chemical Company (St. Louis, U.S.A.). Peroxidase-conjugated sheep anti-rabbit immunoglobulin G (IgG) was obtained from Organon Teknika Corp. (Philadelphia, U.S.A.). Unless otherwise directed, all reagents were of analytical grade and obtained from Wako Pure Chemical Co. (Osaka, Japan).

Purification and Characterization of α -GalNAcases The purification of α -GalNAcase I and α -GalNAcase II from squid liver, their enzymatic activities and isoelectric points were determined by the previously described procedures.^{16,18)}

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified enzymes was performed using 10% gel in Tris-glycine buffer (pH 8.3) according to the procedure described by Laemmli.¹⁹⁾ Gels were stained for protein with 0.25% Coomassie brilliant blue R-250 in 12.5% acetic acid and then destained with 5% acetic acid.

Peptide Mapping α -GalNAcase I or α -GalNAcase II (50 μ g) was solubilized in 8 M urea and diluted to 2 M urea with 0.05 M Tris-HCl buffer, pH 8.5. To the solution, TPCK-treated trypsin was added at a final protein to enzyme weight ratio of 50:1 and incubated at 37 °C for 24 h. The reaction mixture was immediately subjected to reversed-phase high performance liquid chromatography (RP-HPLC) separation on a C4 column (4.6 $\times$ 150 mm) at a flow rate of 1 ml/min followed by elution with a 60 min linear gradient of CH₃CN (5–80%) in 0.1% trifluoroacetic acid (TFA). Absorbance was monitored by UV detection at 215 nm.

Production of Antisera and Western Blotting Antibody against squid α -GalNAcase I and α -GalNAcase II were obtained by the immunization of New Zealand white rabbits. The antisera were purified by column chromatography using protein G-Sepharose (Amersham Biosciences, U.S.A.) and used for Western blot analysis.

Purified α -GalNAcase I and α -GalNAcase II were separated by SDS-PAGE and then transferred electrophoretically to Immobilon-P membrane (Millipore, U.S.A.). Non-specific binding sites were saturated by incubation with 3% blocking solution (bovine serum albumin Fr. V) in 0.05 M Tris-HCl, 150 mM sodium chloride and 0.1% sodium azide (Buffer A) for 1 h at room temperature. The membrane was then incubated with primary antibody at an appropriate dilution for 30 min. After rinsing, the membrane was incubated for 1 h with peroxidase-conjugated sheep anti-rabbit IgG diluted 1:1000 in buffer A. The protein which crossreacted with the antibody was visualized using 3,3'-diaminobenzidine intensified with 0.001% hydrogen peroxide.

Surface Plasmon Resonance (SPR) Spectroscopy Real time interaction of α -GalNAcase I or α -GalNAcase II with the anti- α -GalNAcase I or anti- α -GalNAcase II antibody was performed by SPR spectroscopy using the BIAcore 2000 (Biacore, Inc., Uppsala, Sweden). Antibody was covalently

immobilized on a flow cell (FC2) of a CM5 sensor chip by amine coupling according to the manufacturer's instruction. A blank surface (FC1) was made by ethanolamine deactivation of the activated dextran surface. All measurements were performed using HBS-EP (10 mM Hepes pH 7.4, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.005% (v/v) Surfactant P20). Purified α -GalNAcase I and α -GalNAcase II were each diluted in HBS-EP buffer to 20 μ g/ml and injected over the flow cells at a 10 μ l/min for 180 s. The bound analytes were removed by washing the buffer for 60 s. The first flow cell (FC1) was the minus protein control channel and was subtracted from the sample channel (FC2) during the run to obtain the overlay sensogram.

Amino Acid Sequence The N-terminal amino acids of the enzymes were analyzed with a fully automated gas-phase protein sequencer (Shimadzu Corporation Ltd., Kyoto, Japan).

RESULTS AND DISCUSSION

Extraction and purification of α -GalNAcase I and α -GalNAcase II from squid liver were performed by the methods as previously described.¹⁶⁾ Figure 1 shows the purified α -GalNAcase I and α -GalNAcase II on SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie blue. Both α -GalNAcase I and α -GalNAcase II enzymes appeared to be homogeneous and each gave a single protein band with molecular masses of 48 kDa and 43 kDa, respectively. Isoelectric focusing of α -GalNAcase I and α -GalNAcase II displayed single peaks with a pI of 4.7 and 5.2, respectively. Consistent with the previous findings,¹⁶⁾ α -GalNAcase I contained α -galactosidase activity (7% of α -*N*-acetylgalactosaminidase activity) toward *p*-nitrophenyl- α -galactoside as a substrate, while α -GalNAcase II was almost devoid of such activity. No other activity for example, β -galactosidase, α - or β -glucosidase, α -L-fucosidase, α -mannosidase, or β -*N*-acetylhexosaminidase activity, was observed either.

Chemical comparison between α -GalNAcase I and α -GalNAcase II were carried out first by N-terminal sequencing of the first twenty amino acids. Table 1 summarizes the deduced amino acid sequences of α -GalNAcase I and α -GalNAcase II and compares with the corresponding sequences from human and chicken α -GalNAcases.^{10,20)} Analysis of sequences revealed 80% homology between α -GalNAcase I

Fig. 1. SDS-PAGE of Purified α -GalNAcase I and II from Squid Liver

2 μ g of each enzyme was resolved by 10% SDS-PAGE and the gel was stained with Coomassie brilliant blue. Lane 1, molecular weight marker; lane 2, α -GalNAcase I; lane 3, α -GalNAcase II.

Table 1. N-Terminal Amino Acid Sequences of the Squid Liver α -GalNAcase I and α -GalNAcase II

Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Homology
Human α -GalNAcase	L	D	N	G	L	L	Q	T	P	P	M	G	W	L	A	W	E	R	F	R	100%
Chicken α -GalNAcase	L	E	N	G	L	A	R	T	P	P	M	G	W	L	A	W	E	R	F	R	85%
Squid α -GalNAcase I	L	N	N	G	L	A	L	T	P	P	M	G	W	L	S	W	E	R	F	R	80%
Squid α -GalNAcase II	L	D	N	G	L	M	R	T	P	P	M	G	W	L	V	W	E	R	F	R	85%

and α -GalNAcase II. Both enzymes were also highly homologous (80–85%) to human and chicken α -GalNAcases. The significant homology between α -GalNAcase I and α -GalNAcase II indicated that they are very similar in their primary structure.

Peptide mapping is a common method for comparing the protein primary structures and very useful for demonstrating protein identity. To compare α -GalNAcase I with α -GalNAcase II by peptide mapping, both the enzymes were digested with trypsin and the resulting digests were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). The chromatograms are shown in Fig. 2. Approximately 13–16 major peptide peaks were generated in each protein and 6–8 peptides were found apparently to be overlapped. The overlapped peptides differed in concentration possibly due to the differences in sensitivity of the proteins to trypsin proteolysis. We could not confirm the peptides by determining the amino acid sequence due to either low yield or contamination with the neighboring peptides. The remaining non-overlapping peptides could be accounted for the differences in primary structure of α -GalNAcase I and α -GalNAcase II. Taken together, our protein chemical data suggest that α -GalNAcase I and α -GalNAcase II are closely related with regard to primary structure.

In a second approach to study structural homologies of α -GalNAcase I and α -GalNAcase II, immunological techniques were applied using polyclonal antibodies that were raised in rabbits against the purified α -GalNAcase I and α -GalNAcase II. Western blot results (Fig. 3A) showed that anti- α -GalNAcase I antibody reacted with α -GalNAcase I, but not with α -GalNAcase II. As expected, the same results were obtained when tested with anti-human placental α -GalNAcase antibody produced in our laboratory (data not shown).¹⁸⁾ In contrast, anti- α -GalNAcase II antibody reacted with both α -GalNAcase I and α -GalNAcase II. Surface plasmon resonance (SPR) spectroscopy is a sensitive and modern technique to monitor the protein–protein interaction. To rule out the possibility of any artifact in Western blot, the molecular interaction of α -GalNAcase I or α -GalNAcase II with the anti- α -GalNAcase I or α -GalNAcase II antibody was further examined by SPR spectroscopy. As shown in Fig. 3B, anti- α -GalNAcase I did not bind with α -GalNAcase II, but bound with α -GalNAcase I. Whereas anti- α -GalNAcase II bound with both α -GalNAcase I and α -GalNAcase II. The nonreactivity of α -GalNAcase II with anti- α -GalNAcase I indicates the absence of an epitope in α -GalNAcase II and suggest the differences in conformation between α -GalNAcase I and α -GalNAcase II. Whereas, the reactivity of anti- α -GalNAcase II with both α -GalNAcase I and α -GalNAcase II suggests the presence of common antigenic determinants on both enzymes and indicates their similarity in structure. These re-

Fig. 2. Peptide Maps of α -GalNAcase I and α -GalNAcase II

RP-HPLC separation of trypsin digested peptides of squid α -GalNAcase I (A) and α -GalNAcase II (B). The asterisk marks denote the overlapping peptides. See text for details.

sults, therefore, were in close agreement with the peptide mapping data.

In conclusion, our data presented here suggest that α -GalNAcase I and α -GalNAcase II are closely related with regard to primary and higher order structure. Their structural similarity could account for their similar enzymatic properties such as the hydrolysis of α -galactosaminyl group in glycoproteins and glycolipids as suggested from *in vitro* studies.^{16,17)} On the other hand, the nonhomologous molecular domains might be responsible for their differences in specific action like α -galactosidase activity. Now it will be of interest to find out whether α -GalNAcase I and α -GalNAcase II are derived from the same gene which, by differential splicing, produces two different enzymes or from different genes evolved from a common ancestor.

Acknowledgements We thank Prof. Dr. T. Komiyama, Faculty of Pharmaceutical Science, Niigata University of Pharmacy and Applied Life Sciences, for his advice and comments during the preparation of the peptide map and the sequence analysis. This research was supported in part by a Grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

Fig. 3. Western Blot and Surface Plasmon Resonance (SPR) Analysis for the Reaction between α -GalNAcase I or α -GalNAcase II and Anti- α -GalNAcase I or α -GalNAcase II Antibody

(A) Western blot of α -GalNAcase I and α -GalNAcase II with anti- α -GalNAcase I (left panel) and anti- α -GalNAcase II antibody (right panel). (B) Overlay of SPR sensogram resulting from the injection of α -GalNAcase I or α -GalNAcase II over immobilized anti- α -GalNAcase I (1) or α -GalNAcase II (2) antibody. The arrows represented by analyte and buffer indicate the start of the association and dissociation curves, respectively.

REFERENCES

- Michalski J.-C., Klein A., *Biochim. Biophys. Acta*, **1455**, 69–84 (1999).
- Schindler D., Bishop D. F., Wolfe D. F., Wang A. M., Egge H., Lemieux R. U., *N. Engl. J. Med.*, **320**, 1735–1740 (1989).
- Kanzaki T., Yokota M., Mizuno N., Matsumoto Y., Hirabayashi Y., *Lancet*, **1**, 875–877 (1989).
- Sakuraba H., Matsuzawa F., Aikawa S., Doi H., Kotani M., Nakada H., Fukushima T., Kanzaki T., *J. Hum. Genet.*, **49**, 1–8 (2004).
- Kanekura T., Sakuraba H., Matsuzawa F., Aikawa S., Doi H., Hirabayashi Y., Yoshii N., Fukushima T., Kanzaki T., *J. Dermatol. Sci.*, **37**, 15–20 (2005).
- Zhu A., Monahan C., Wang Z.-K., *Biochim. Biophys. Acta*, **1297**, 99–104 (1996).
- Hsin-Yeh, Chapman L. F., Calcutt M. J., Smith D. S., *Artif. Cells Blood Substit. Immobil. Biotechnol.*, **33**, 187–199 (2005).
- Kint J. A., *Arch. Int. Physiol. Biochem.*, **79**, 633–634 (1971).
- Beutler E., Kuhl W., *J. Biol. Chem.*, **247**, 7195–7200 (1972).
- Tsuji S., Yamauchi T., Hiraiwa M., Isobe T., Okuyama T., Sakimura K., Takahashi Y., Nishizawa M., Uda Y., Miyatake T., *Biochem. Biophys. Res. Commun.*, **163**, 1498–1504 (1989).
- Wang A. M., Bishop D. F., Desnick R. J., *J. Biol. Chem.*, **265**, 21859–21866 (1990).
- Uda Y., Li S.-C., Li Y.-T., McKibbin J. M., *J. Biol. Chem.*, **252**, 5194–5200 (1977).
- Sung S. S. J., Sweely C. C., *J. Biol. Chem.*, **255**, 6589–6594 (1980).
- Callahan J. W., Lassila E. L., Tandt W. D., Phillipart M., *Biochem. Med.*, **7**, 424–431 (1973).
- Levy G. N., Aminoff D., *J. Biol. Chem.*, **255**, 11737–11742 (1980).
- Itoh T., Uda Y., *J. Biochem.*, **95**, 959–970 (1984).
- Itoh-Nashida T., Shiraishi T., Uda Y., *Chem. Pharm. Bull.*, **36**, 4000–4007 (1988).
- Hiraiwa M., Nishizawa M., Uda Y., Nakajima T., Miyatake T., *J. Biochem.*, **103**, 86–90 (1988).
- Laemmli U. K., *Nature (London)*, **227**, 680–685 (1970).
- Zhu A., Goldstein J., *Gene*, **137**, 309–314 (1993).