Identification of a Novel Chondroitin Hydrolase in Caenorhabditis elegans

Received for publication, November 9, 2007, and in revised form, March 25, 2008 Published, JBC Papers in Press, April 4, 2008, DOI 10.1074/jbc.M709236200

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Hyaluronidases have been postulated to be the enzyme acting at the initial step of chondroitin sulfate (CS) catabolism in vivo. Since chondroitin (Chn) but not hyaluronic acid (HA) has been detected in Caenorhabditis elegans, the nematode is a good model for elucidating the mechanism of the degradation of CS/Chn in vivo. Here we cloned the homolog of human hyaluronidase in C. elegans, T22C8.2. The Chn-degrading activity in vitro was first demonstrated when it was expressed in COS-7 cells. The enzyme cleaved preferentially Chn. CS-A and CS-C were also depolymerized but to lesser extents, and HA was hardly degraded. In order of preference, the substrates ranked Chn ≫ CS-A > CS-C > HA. The products of the degradation of Chn by the enzyme were characterized by anion-exchange high performance liquid chromatography and delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The structure of the major component in the digest was determined as GlcUA1-3GalNAcβ1-4GlcUAβ1-3GalNAc, where GlcUA and GalNAc represent β-glucuronic acid and N-acetyl-β-galactosamine, respectively, indicating that this enzyme is a Chn hydrolase, an endo-β-galactosaminidase specific for Chn. Investigation of the effects of pH on the activity revealed the optimum pH of Chn hydrolase to be 6.0. Since Chn in C. elegans has been demonstrated to play critical roles in cell division, Chn hydrolase possibly regulates the function of Chn in vivo. This is the first demonstration of a Chn hydrolase in an animal.

Chondroitin sulfate proteoglycans (CS-PGs) are ubiquitous components of the extracellular matrix of connective tissues and are also found at the surface of many cell types (1, 2). They are involved in the regulation of various biological processes such as cell proliferation, differentiation, and migration, cell-cell recognition, extracellular matrix deposition, and tissue morphogenesis (3–6). CS chains have a linear polymer structure composed of the repeating disaccharide unit –4GlcUA1-3GalNAc1–, where GlcUA represents α-glucuronic acid and GalNAc represents N-acetyl-β-galactosamine, which are sulfated at different positions in various combinations (1, 4).

Recently, it has been demonstrated that chondroitin (Chn), which has no sulfate-related modifications, is involved in cell division during early development in Caenorhabditis elegans (7–9). When Chn synthase or Chn-polymerizing factor in C. elegans is knocked down using RNA-mediated interference, most oocytes and fertilized eggs die in utero after an oscillation of cell division and reversion of cytokinesis (7–9). Enzymatic degradation of cell surface Chn using an exogenously added bacterial chondroitinase also resulted in similar effects on cell division (8). Thus, the mechanism behind the biosynthesis of Chn and CS has attracted much attention and has been investigated in detail by several groups (10, 11), but that of CS catabolism is not well understood. CS is catabolized by the endolytic cleavage of long polysaccharide chains, and then their further degradation is mediated by sulfatases and exoglycosidases from the nonreducing end (12). Although no endoglycosidase specific for CS has been reported, hyaluronic acid (HA)-degrading enzymes, hyaluronidases, are considered to be the enzyme acting at the initial stage of the degradation process (12).

Since Chn but not HA has been detected in C. elegans (13), the nematode is a good model for elucidating the mechanism by...
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which Chn is degraded in vivo. Unexpectedly, we have identified a homolog of human hyaluronidase in the C. elegans genome. Chn and HA differ in structure only in the configuration at the C-4 position of the hexosamine residue. Thus, the gene may encode a Chn-degrading enzyme. In this study, we examined the activity of the recombinant protein to depolymerize Chn and demonstrated that the gene product hydrolyzes Chn, and to a much lesser extent, HA.

EXPERIMENTAL PROCEDURES

Materials—The following sugars and enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): CS-A from whale cartilage, CS-C from shark cartilage, Chn, a chemically desulfated derivative of CS-A, heparan sulfate from bovine kidney, eight unsaturated standard disaccharides derived from CS and HA, and chondroitinase (CSase) AC-II from Arthrobacter aurescens (EC 4.2.2.5.). HA from human umbilical cord was obtained from Sigma. HA preparations having an average molecular mass of 980, 132, and 35 kDa, which were produced by microbial fermentation of Streptococcus pyogenes, were from R&D Systems, Inc. (Minneapolis, MN). The average molecular size of the commercial Chn, CS-A, CS-C, and HA from human umbilical cord was determined to be 23, 34, 64, and 1,000 kDa, respectively, by gel filtration chromatography with size-defined dextran preparations as standards (data not shown). COS-7 cells were purchased from the Japan Health Sciences Foundation (Tokyo, Japan). The Superdex™ peptide HR10/30 column, and prepacked disposable PD-10 columns containing Sephadex G-25 (medium) were obtained from Amersham Biosciences. 2-Aminobenzamide (2AB) derivatives of authentic saturated tetrasaccharides, GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S)-2AB, and GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S)-2AB, where 4S and 6S represent 4- and 6-Aminobenzamide (2AB) derivatives of authentic saturated (medium) were obtained from Amersham Biosciences.

Preparation of Fluorescein 5(6)-Isothiocyanate (FITC)-labeled Chn—(2 mg) was incubated with 1 mg of FITC dissolved in 400 μl of 0.1 M sodium carbonate buffer, pH 9.5, and stirred at 4 °C overnight in darkness (17). To remove excess FITC-labeling reagents, the reaction mixture was subjected to gel filtration on a PD-10 column with phosphate-buffered saline as the eluent.

Molecular Cloning of T22C8.2—A tBLASTn analysis of the GenBank™ database, using the sequences of human hyaluronidases, identified a highly homologous clone, T22C8.2 (GenBank™ accession number NM_063429) in the C. elegans genome. The cDNA sequence was obtained using WormBase (accession number WBGene 00011923). Since Chn but not HA was found in C. elegans, this sequence was predicted to encode a Chn hydrolase (Chnase).

Cloning of C. elegans Chnase cDNA—The putative full-length open reading frame encoding a presumed Chnase was amplified from C. elegans N2 cDNA by two rounds of PCR using specific primers corresponding to the sequences in the 5′- and 3′-noncoding regions. The first PCR was performed with the primers, 5′-CTC GAG TGG GTT GAA GTT TGG TAG and 3′-CTG GAG TCT CAA AGT CAA AAT GGC AAA-3′. The second PCR was performed with the nested primers, 5′-CTC GAG GGC AAA TAA AGC TTG ATC CAA-3′ and 5′-CTC GAG AGA AGA TTG CAC TGC CAA CA-3′. Each PCR was carried out with KOD-Plus DNA polymerase (Toyobo, Tokyo, Japan) in the presence of 5% (v/v) dimethyl sulfoxide for 30 cycles at 95 °C for 30 s, 54 °C for 45 s, and 68 °C for 2 min. The amplified cDNA fragment of expected size (~1.4 kb) was subcloned into a pGEM®-T Easy vector (Promega, Tokyo) and sequenced in a CEQ 8000 DNA sequencer (Beckman Coulter).

Construction of an Expression Vector Containing a cDNA Fragment Encoding a Soluble Form of Chnase—The DNA fragment, which encodes the putative Chnase protein lacking the first N-terminal 26 amino acids, the predicted transmembrane region, was amplified by PCR with the pGEM®-T Easy vector containing the full-length form of Chnase as a template, using a 5′ primer containing an in-frame BamHI site (5′-GCC GAT CCG GTT CGG CCT CC-3′) and a 3′ primer containing a BamHI site located 19 bp downstream from the stop codon (5′-GCC GAT CCT CTT GTT CAC TAT TAT-3′). PCR was carried out with KOD-Plus DNA polymerase for 30 cycles at 95 °C for 30 s, 55 °C for 45 s, and 68 °C for 2 min. The amplified PCR fragment was subcloned into the BamHI site of the expression vector p3XFLAG-CMV-8 (Sigma), resulting in the fusion of Chnase to the preprotrypsin leader sequence and the 3XFLAG tag sequence present in the vector.

Expression of a Soluble Form of Chnase—The expression plasmid (6.6 μg) was introduced into COS-7 cells using FuGENETM6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. After 3 days of culture at 37 °C, 10 ml of the culture medium was collected and incubated with 1 ml of anti-FLAG® M2 affinity gel (Sigma) at 4 °C overnight. The resin was washed with 25 mM Tris-buffered saline containing 0.05% Tween 20 (TBST), and bound proteins were eluted with 5 ml of 100 μg/ml of the FLAG-peptide (Sigma). The eluates were incubated with the SDS sample buffer and dithiothreitol solution (New England Biolabs, Ipswich, MA) in a total volume of 30 μl at 100 °C for 5 min and subjected to SDS-PAGE. Proteins were resolved on 7.5% SDS-polyacrylamide gels and visualized by silver staining or transferred to a polyvinylidene difluoride membrane. The membrane was incubated with anti-FLAG® M2 monoclonal antibody (Sigma) diluted 1:1000 with TBST overnight and then with the ECL™ horseradish peroxidase-labeled anti-mouse IgG antibody (GE Healthcare) diluted 1:10,000 with TBST. The bound antibody was detected using an ECL Advance western blotting detection kit (GE Healthcare).

Measurement of the Enzyme Activity—The cells transfected with T22C8.2 were cultured for 2 days, and 3 ml of the medium was purified with 15 μl of anti-FLAG® M2 affinity gel at 4 °C overnight. The resin was washed with TBST and subsequently with 50 mM phosphate, pH 6.0, containing 150 mM NaCl, and then resuspended in 4 μl of the same buffer containing ~6 μg of the FITC-labeled Chn. The mixture was incubated at 28 °C for 12 h. The resin was removed by filtration using Ultrafree-MC (Durapore polyanionide difluoride 0.45 μm) (Millipore), and the sample solution was subjected to gel filtration chromatog-
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An acceptor, and 10 national formulary units of testicular hyaluronidase were incubated (1 national formulary unit corresponds to the amount of the enzyme which hydrolyzes 74 μg of hyaluronate/min).

Characterization of the Reducing Terminal Sugar Residue of the Degradation Products—The 2AB-derivative of the degradation products of Chn was digested with CSase AC-II (5 mIU) in 50 mM sodium acetate buffer, pH 6.0 (20). The digest was analyzed by anion-exchange HPLC on an amine-bound silica PA03 column as described above.

Delayed Extraction Matrix-assisted Laser Desorption Ionization Time-of-flight (DE MALDI-TOF) MS—The 2AB-labeled oligosaccharides fractionated by gel filtration HPLC on a Superdex peptide column were collected and individually analyzed by DE MALDI-TOF MS in the linear mode by Voyager-DE STR-H (Applied Biosystems, Foster City, CA) (21, 22). Each sample (1 pmol) was mixed with a matrix, 2,5-dihydroxybenzoic acid, on a sample plate well, dried under an air stream, and then analyzed by MS in the negative or positive mode.

RESULTS

Molecular Cloning of T22C8.2—Screening of the nonredundant data base at the NCBI, National Institutes of Health (Bethesda, MD), using the deduced amino acid sequences of human hyaluronidases, identified a homolog designated T22C8.2 (WormBase accession T22C8.2) in C. elegans (Fig. 1). It contained a 5′-untranslated region of 151 bp, a single open reading frame of 1,377 bp coding for a protein of 458 amino acids with four potential N-glycosylation sites, and a 3′-untranslated region of 84 bp. The SOSUI system, a web site for predicting the secondary structure of membrane proteins from protein sequences, revealed a prominent hydrophobic segment of 23 amino acid residues in the N-terminal region of T22C8.2, predicting that the protein has a type II transmembrane topology. Data base searches suggested that the amino acid sequence displayed 28, 27, 27, 27, and 28% identity with human hyaluronidases 1, 2, 3, and 4 and PH-20, respectively (Fig. 1). Thus, the structural features of the identified protein sequence suggest that the gene product of T22C8.2 is involved in the degradation of HA. However, C. elegans produces Chn but not HA (13). Hence, the protein encoded by T22C8.2 may have Chn-degrading activity and play a crucial role in the metabolism of Chn in C. elegans. Thus, the putative catalytic activity of a recombinant form of T22C8.2 was investigated.

Demonstration of the Chn-degrading Activity—To facilitate the functional analysis of Chnase, a soluble form of the protein was generated by replacing the putative signal sequence with a cleavable preprotrypsin leader sequence and a 3XFLAG tag fusion protein as described under “Experimental Procedures.” The soluble protein was expressed in COS-7 cells at 37 °C as a recombinant protein fused with the 3XFLAG tag. The fusion protein secreted in the medium was adsorbed onto an anti-FLAG® M2 affinity gel for the elimination of endogenous glycosidases, and then the protein-bound resin was used as an enzyme source. The purity of the enzyme was examined by western blotting as well as silver staining. Upon the silver staining, in contrast to the sample from mock-transfected cells, almost a single induced band was discernible at a molecular
### A

| Chnase | HYAL1 | HYAL2 | HYAL3 | HYAL4 | PH-20 |
|--------|-------|-------|-------|-------|-------|
| VTDYVHKGLLVLLFTIIGAAGKYYGSAGAQHTTVVYKGVVTCT--EVSAYEYKGLQWGQFA | CAGAGATCAVLITLMAQFGQPLPP--NRPQNTMOLRKHGVVDPQTVWVAVQHQT--PM | CAGAGATCAVLITLMAQFGQPLPP--NRPQNTMOLRKHGVVDPQTVWVAVQHQT--PM | CAGAGATCAVLITLMAQFGQPLPP--NRPQNTMOLRKHGVVDPQTVWVAVQHQT--PM | CAGAGATCAVLITLMAQFGQPLPP--NRPQNTMOLRKHGVVDPQTVWVAVQHQT--PM | CAGAGATCAVLITLMAQFGQPLPP--NRPQNTMOLRKHGVVDPQTVWVAVQHQT--PM |
| 1      | 1     | 1     | 1     | 1     | 1     |

### B

**FIGURE 1. Comparison of the putative Chnase with reported human hyaluronidases.** A, multiple sequence alignment of *C. elegans* Chnase and human hyaluronidases using ClustalW multiple sequence alignment program (version 1.83). Introduced gaps are shown by hyphens, and aligned identical residues are boxed (black) for all sequences and dark gray for four or five sequences. Four potential N-glycosylation sites for Chnase are indicated by asterisks. The hydrophobic amino acid sequence as a putative transmembrane region or a signal peptide sequence in Chnase is shown by a *squared box.* B, phylogenetic tree produced using the ClustalW algorithm. The length of each *horizontal* line is proportional to the degree of divergence in the amino acid sequence.
mass of 80 kDa (supplemental Fig. 1A), although the expression level was low. The band with the same molecular size was clearly detected by western blotting under reducing and nonreducing conditions (supplemental Fig. 1, B and C). Since the expected molecular mass of this polypeptide is 53 kDa, the recombinant protein detected by western blotting seems to be posttranslationally modified, presumably glycosylated.

The bound fusion protein was assayed for Chn-degrading enzyme activity at 28 °C for 12 h using FITC-Chn as a substrate, and the digest was analyzed by gel filtration HPLC on a Superdex peptide column (Fig. 2). FITC-Chn was efficiently degraded into oligosaccharides (Fig. 2B). No detectable catalytic activity was observed for the control sample prepared from a conditioned medium of mock-transfected cells. Thus, Chn-degrading activity was demonstrated for T22C8.2. Note that the oligosaccharides detected with FITC at the carboxyl groups of some GlcUA residues and that elution of FITC-labeled oligosaccharides was retarded by hydrophobic interactions of FITC with the resin. Therefore, the positions of FITC-labeled Chn oligosaccharides could not be calibrated.

The effect of temperature on the Chnase activity was examined by incubating at 15, 20, or 28 °C. With the activity at 28 °C taken as 1.0, the relative activity detected at 15 or 20 °C was 0.5 or 0.7, respectively. The following experiments were conducted by incubating at 28 °C.

**Mechanism of Degradation of Chn—** To investigate the Chn-degrading activity of T22C8.2 in more detail, the enzyme digest of Chn was labeled with 2AB (see "Experimental Procedures") and subjected to gel filtration HPLC on a Superdex peptide column (Fig. 3A). Two major peaks, O-1 and O-2, were eluted at the positions of the authentic nonsulfated tetra- and hexasaccharides, respectively. To characterize the structure of the digests, O-1 and O-2 were collected and subjected to DE MALDI-TOF MS.

DE MALDI-TOF MS of the 2AB-derivatized samples in the positive and negative ion mode defined their molecular weights, from which the sugar composition and the number of O-sulfate groups present in each fraction were inferred. In the positive and negative ion mode DE MALDI-TOF MS spectrum of fraction O-1, molecular ion signals were observed as \([M + H]^+\), \([M + Na]^+\), and \([M - H]^–\) at \(m/z\) 897, 919, and 895, respectively (Fig. 4A, Table 1), suggesting that the component in this fraction was HexUA\(_2\)HexNAC\(_2\)-2AB, where HexUA and HexNAC represent hexuronic acid and N-acetylgalactosamine, respectively. In the DE MALDI-TOF MS spectrum of fraction O-2, molecular ion signals were observed as \([M + H]^+\), \([M + Na]^+\), \([M + K]^+\), and \([M - H]^–\) at \(m/z\) 1,277, 1,299, 1,315, and 1,274, respectively (Fig. 4B, Table 1), suggesting that the component in this fraction was HexUA\(_2\)HexNAC\(_2\)-2AB. Thus, the major components in fractions O-1 and O-2 were assumed to contain saturated tetra- and hexasaccharides, respectively, indicating that the enzyme is not an eliminase but a hydrolase. Hence, the enzyme was identified as Chn hydrolase.

To investigate whether the enzyme is a hexosaminidase or glucuronidase, the terminal sugar residue of the oligosaccharides in the enzyme digest was analyzed. The digest was labeled with 2AB and analyzed by anion-exchange HPLC before and after digestion with CSase AC-II (Fig. 5). Before the digestion, peaks were eluted at the positions corresponding to those of the...
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2AB-derivatives of the authentic Chn tetra-(GlcUA-GalNAc-GlcUA-GalNAc), hexa-(GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc), and octasaccharide (GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc-GlcUA-GalNAc), respectively, which were produced by digestion with testicular hyaluronidase. After the digestion with CSase AC-II, peaks were eluted at the position of the unsaturated disaccharide, ΔHexUA-GalNAc-2AB, indicating that the reducing terminal sugar residue of the products of digestion by Chnase is GalNAc and that Chnase is a hexosaminidase, not a glucuronidase.

Substrate Specificity of Chnase—To characterize the specificity of Chnase, CS-A, CS-C, dermatan, heparan sulfate, HA from human umbilical cord, and HA preparations from S. pyogenes (an average molecular mass of 980, 132, and 35 kDa) were used as substrates. The incubation mixtures were analyzed by gel filtration HPLC on a Superdex peptide column after labeling with 2AB to detect the newly formed reducing ends (Fig. 3). The chromatograms showed that Chnase had activity to degrade CS-A (Fig. 3B), CS-C (Fig. 3C), and HA (Fig. 3D), but dermatan and heparan sulfate were not depolymerized (data not shown). No difference was observed in the amount of oligosaccharides formed among the HA preparations used, suggesting that the chain length of HA does not affect the susceptibility to the enzyme. Although the same amount of each substrate was used under the same conditions, the amounts of the products generated by the digestion were different. Chn was almost completely broken down into tetra- and hexasaccharides (Fig. 3A), whereas CS-A, CS-C, and HA were degraded to a lesser extent. The relative rates of the degradation of these polysaccharides suggested that in order of preference by Chnase, the substrates ranked as follows: Chn >> CS-A > CS-C >> HA.

To clarify whether Chnase recognizes and acts on the nonsulfated or sulfated structure in the CS chains, the peaks A-1 and C-1 (Fig. 3, B and C) generated from CS-A and CS-C, respectively, were fractionated and then analyzed by anion-exchange HPLC and MALDI-TOF MS. The chromatograms showed that the major component in fractions A-1 and C-1 was eluted at the position of the 2AB-derivative of the authentic standard tetrasaccharide, GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S) (GlcUA-GalNAc(6S)-GlcUA-GalNAc(6S), respectively (Fig. 6, A and B), suggesting that Chnase cleaves the sulfated galactosaminidic bonds in CS-A and CS-C, namely GalNAc(4S)-GlcUA and GalNAc(6S)-GlcUA. The negative mode DE MALDI-TOF MS of fractions A-1 and C-1 showed the molecular ion signal at $m/z$ 1,077 to be $[M + Na - 2H]^{-}$, indicating that the component in these fractions was HexUA$_2$HexNAc$_2$(OSO$_3$)$_2$-2AB, consistent with the results of HPLC described above.

The Optimum pH for the Chnase Activity—The effect of pH on the Chnase activity was examined by incubating the Chnase-bound resin with FITC-labeled Chn over a range of pH values from 4.5 to 8.0 (Fig. 7). The results indicated the optimum pH of Chnase to be 6.0.

Kinetic Analysis of Chnase—The initial reaction rates and substrate concentrations (as disaccharide) were used for kinetic analysis by Lineweaver-Burk plot (supplemental Fig. 2). The apparent Michaelis-Menten constants as well as $V_{max}$ for Chn, CS-A, and HA from S. pyogenes (35 kDa) were determined as 0.12, 0.48, and 49.3 mmM as well as 3.8, 1.2, and 0.4 pmol/min, respectively (Table 2).

Transglycosylation Activity—Since testicular hyaluronidase catalyzes transglycosylation as well as hydrolysis on HA (19), transglycosylation activity of nematode Chnase was examined by incubating with Chn as a donor and 2AB-labeled Chn-hexasaccharide as an acceptor. However, 2AB-labeled oligosaccharides longer than hexasaccharide were not detected under the conditions used (results not shown), although the transglycosylation activity by sheep testicular hyaluronidase was confirmed. Thus, Chnase has no or little, if any, transglycosylation activity.

### Table 1
Assignment of molecular ion signals afforded by positive and/or negative DE MALDI-TOF MS of the 2AB-derivatized products of the degradation of Chn, CS-A, CS-C, and HA by the C. elegans Chnase.

| Fraction | $[M + H]^+$ | $[M + Na]^+$ | $[M + K]^+$ | $[M - H]^-$ | $[M + Na - 2H]^-$ | Molecular composition of M |
|----------|-------------|--------------|-------------|-------------|-------------------|--------------------------|
| O-1      | 897         | 919          | 1299        | 1315        | 1274              | HexUA$_2$HexNAc$_2$-2AB  |
| O-2      | 1277        | 1299         | 1315        | 1274        | 1277              | HexUA$_2$HexNAc$_2$-2AB  |
| A-1      | 897         | 919          | 1277        | 1315        | 1274              | HexUA$_2$HexNAc$_2$(OSO$_3$)$_2$-2AB |
| C-1      | 897         | 919          | 1277        | 1315        | 1274              | HexUA$_2$HexNAc$_2$(OSO$_3$)$_2$-2AB |

FIGURE 4. Identification of the Chnase digestion products by DE MALDI-TOF MS. DE MALDI-TOF MS of the major products of the digestion of Chn with purified Chnase, fractions O-1 (A) and O-2 (B) in Fig. 3A, was recorded in the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as indicated in the figure.
DISCUSSION

In the present study, we demonstrated the Chn hydrolase activity for the C. elegans T22C8.2 protein and characterized its specificity. Although the clone T22C8.2 was found as the C. elegans ortholog of human hyaluronidase, it was not a hyaluronidase but a Chn hydrolase; the enzyme much prefers Chn to HA, and only Chn, no HA, is detected in C. elegans (13). This is the first report of a Chn-degrading enzyme in a metazoan or an animal. Although several CSases have been isolated from bacteria, they are not hydrolases but eliminases (23–25). These bacterial enzymes digest CS as a source of nutrients. Since typical CS-PGs are not synthesized in protozoa, bacterial enzymes are not involved in the in vivo metabolism of structural components of protozoa. Hyaluronidases from vertebrate tissues and the venom of snakes, bees, spiders, and stonefish can depolymerize both HA and Chn/CS and are known as spreading factors (26). These hyaluronidases digest Chn/CS more slowly than HA (27, 28), and their genuine substrate is HA rather than Chn/CS. Thus, these hyaluronidases should not be termed Chnases. The enzyme reported here is the first Chn hydrolase to be identified.

Although Csoka et al. (27) described hyaluronidase-4 as a CS-specific enzyme based on the preliminary evidence, the actual data and conditions for the activity detection were not
presented. To test the CS-specific activity of the enzyme, we also tried to characterize human hyaluronidase-4. Expression of the protein in HEK293 cells was successfully detected by western blotting, but no enzymatic activity was determined under the conditions used in our hands (data not shown). Hyaluronidase-4 might be complexed with CD44 or some other molecule(s) to exert its function.

As shown in Fig. 3, Chnase preferred Chn to CS-A and CS-C as a substrate, indicating that sulfate groups may have negative effects against the enzyme activity, in contrast to bacterial CSase, which prefers CS to Chn (23). Dermatan was not digested by Chnase, and HA was a much less preferred substrate than Chn (Fig. 3), suggesting that Chnase distinguished the steric configuration of the C-5 position of uronic acid residues as well as that of the C-4 position of hexosamine residues. Based on the kinetic analysis of Chnase, Michaelis-Menten constants toward Chn, CS-A, and HA were calculated and compared. An apparent $K_m$ value toward HA is ~400 times higher than that toward Chn, indicating that Chn is a much better substrate than HA (Table 2). This preference for the substrate is in contrast to that of hyaluronidases such as human hyaluronidase-1 and PH-20, which cleave HA more efficiently than CS (27, 28). No hydrolases that catalyze CS more efficiently than HA have been reported. Therefore, Chnase is a useful experimental tool with which to depolymerize Chn chains in tissues in contrast to that of hyaluronidase such as human hyaluronidase-4 and PH-20, which cleave HA more efficiently than CS in contrast to that of hyaluronidase such as human hyaluronidase-4 and PH-20, which cleave HA more efficiently than CS in contrast to that of hyaluronidase such as human hyaluronidase-4 and PH-20, which cleave HA more efficiently than CS.

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In vertebrates, the catabolism of CS is a highly ordered process dependent upon the actions of specific glycosidases and sulfatases in lysosomes (12). Degradation is initiated by the endolytic cleavage of the long polysaccharide chain into smaller fragments. Then actions by exoglycosidases and sulfatases mediate further degradation of the fragments from the nonreducing terminus. Although a series of exoglycosidases and sulfatases have been identified and characterized (12), the endoglycosidase involved at the initial step in the catabolism of CS has not been elucidated. Hyaluronidases can set the stage for the degradation process (12), but no endoglycosidase specific to CS has been reported. All human hyaluronidases showed a similar degree of homology to Chnase, in the range of 27–28% similarity at the amino acid level. Although Csokath et al. (27) suggested hyaluronidase-4 as a CS-specific enzyme, hyaluronidase-4 is not the most homologous to Chnase (Fig. 1). Thus, it remains to be elucidated whether hyaluronidase-4 is indeed specific to CS. In any case, hyaluronidase-4 is not the only candidate for the enzyme involved in the catabolism of CS because its expression is not ubiquitous but is restricted to the placenta and skeletal muscle (27).

When C. elegans was treated with dsRNA of Chn synthase or Chn-polymerizing factor, most oocytes and fertilized eggs died in utero. Fertilized eggs laid within 11–12 h of the RNA-mediated interference treatment could not complete cell division, particularly cytokinesis (7–9). These results demonstrated the critical roles of Chn in C. elegans. Although its function in vivo has not been clarified, Chnase may well regulate the function of Chn. The Chnase protein possesses a prominent hydrophobic segment in the N-terminal region and appears to be a transmembrane molecule with a type II topology. However, it remains to be investigated whether this enzyme occurs and functions on the cell surface or in the lysosome. Investigation of the cellular distribution of this enzyme in the nematode is in progress.

Güerardel et al. (34) isolated Chn tri-, tetra-, and pentasaccharides from C. elegans after alkaline reductive degradation and analyzed their structure by 400 MHz 1H NMR spectroscopy. Since Chn chains in C. elegans are covalently attached to core proteins through the common tetrasaccharide sequence GlcUA-Gal-Gal-Xyl (35, 36), the Chn oligosaccharides do not seem to be released directly from the core proteins but rather derived from free oligosaccharides, which are degradation intermediates. These oligosaccharides are most likely generated by digestion with Chnase in vivo because their reducing terminal sugar residues are GalNAc. Note that HA oligosaccharides liberated by digestion with hyaluronidases have biological activities, such as induction of the transcription of metalloproteases (37), enhancement of tumor cell motility (38), and inhibition of the tumorigenicity of cancer cells (39). A disaccharide derived from CS was also demonstrated to promote recovery of the central nervous system by modulating both neuronal and microglial behavior (40). Thus, the possibility exists that these Chn fragments are produced as functional signaling molecules to regulate cell behavior.

REFERENCES

1. Rodén, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed) pp. 267–371, Plenum Publishing Corp., New York
2. Iozzo, R. V. (1998) Annu. Rev. Biochem. 67, 609–652
3. Silbert, J. E., and Sugumaran, G. (2003) Glycoconjug. J. 19, 227–237
4. Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., and Kitagawa, H. (2003) Curr. Opin. Struct. Biol. 13, 612–620
5. Nandini, C. D., and Sugahara, K. (2006) Adv. Pharmacol. 53, 253–279
6. Sugahara, K., and Mikami, T. (2007) Curr. Opin. Struct. Biol. 17, 536–545
7. Hwang, H. Y., Olson, S. K., Esko, J. D., and Horvitz, H. R. (2003) Nature 423, 439–443
8. Mizuguchi, S., Uyama, T., Kitagawa, H., Nomura, K. H., Dejima, K., Gengyo-Ando, K., Mitani, S., Sugahara, K., and Nomura, K. (2003) Nature 423, 443–448
9. Izumikawa, T., Kitagawa, H., Mizuguchi, S., Nomura, K. H., Nomura, K., Tamura, J., Gengyo-Ando, K., Mitani, S., and Sugahara, K. (2004) J. Biol. Chem. 279, 53755–53761
10. Sugahara, K., and Kitagawa, H. (2000) Curr. Opin. Struct. Biol. 10, 518–527
11. Pavón, M. S., Vilela-Silva, A. C., and Mourão, P. A. (2006) Adv. Pharmacol. 53, 117–140
12. Prabhakar, V., and Saissekharan, R. (2006) Adv. Pharmacol. 53, 69–115
13. Yamada, S., Van Die, I., Van den Eijnden, D. H., Yokota, A., Kitagawa, H., and Sugahara, K. (1999) FEBS Lett. 459, 327–331
14. Sugahara, K., Tanaka, Y., and Yamada, S. (1996) Glycoconjug. J. 13, 609–619
15. Kinoshita, A., and Sugahara, K. (1999) Anal. Biochem. 269, 367–378
16. Mizukami, T., Mizumoto, S., Kago, N., Kitagawa, H., and Sugahara, K. (2003) J. Biol. Chem. 278, 36115–36127
17. Toyoshima, M., and Nakajima, M. (1999) J. Biol. Chem. 274, 24153–24160
18. Kawashima, H., Atarashi, K., Hirose, M., Yamada, S., Sugahara, K., and Miyasaka, M. (2002) J. Biol. Chem. 277, 12921–12930
19. Saitho, H., Takagaki, K., Majima, M., Nakamura, T., Matsu, K., Kasai, M.,...
Narita, H., and Endo, M. (1995) J. Biol. Chem. 270, 3741–3747
20. Sugahara, K., Masuda, M., Harada, T., Yamashina, I., de Waard, P., and Vliegenthart, J. F. G. (1991) Eur. J. Biochem. 202, 805–811
21. Sakaguchi, H., Watanabe, M., Ueoka, C., Sugiyama, E., Taketomi, T., Yamada, S., and Sugahara, K. (2001) J. Biochem. (Tokyo) 129, 107–118
22. Sugiyama, E., Hara, A., Uemura, K., and Taketomi, T. (1997) Glycobiology 7, 719–724
23. Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968) J. Biol. Chem. 243, 1523–1535
24. Hiyama, K., and Okada, S. (1975) J. Biol. Chem. 250, 1824–1828
25. Petit, E., Delattre, C., Papy-Garcia, D., and Michaud, P. (2006) Adv. Pharmacochem. 53, 167–186
26. Frost, G. I., Csoka, T., and Stern, R. (1996) Trends Glycosci. Glycotechnol. 8, 419–434
27. Imanari, T., Shinbo, A., Ochiai, H., Ikei, T., Koshiishi, I., and Toyoda, H. (1992) J. Pharmacobi-Dyn. 15, 231–237
28. Nakagawa, H., Hamayu, Y., Sumi, T., Li, S. C., Maskos, K., Kalayanamitra, K., Mizumoto, S., Sugahara, K., and Li, Y. T. (2007) Glycobiology 17, 157–164
29. Karamanos, N. K., Aletras, A. J., Antonopoulos, C. S., Tsegenidis, T., Tsiganos, C. P., and Vynios, D. H. (1988) Biochim. Biophys. Acta 966, 36 – 43
30. Toyoda, H., Kinohshi-Toyoda, A., and Selleck, S. B. (2000) J. Biol. Chem. 275, 2269–2275
31. Yamada, S., Morimoto, H., Fujisawa, T., and Sugahara, K. (2007) Glycobiology 17, 886 – 894
32. Guérardel, Y., Balanzino, L., Maes, E., Leroy, Y., Coddeville, B., Oriol, R., and Strecker, G. (2001) Biochem. J. 357, 167–182
33. Yamada, S., Okada, Y., Ueno, M., Iwata, S., Deepa, S. S., Nishimura, S., Fujita, M., Van Die, I., Hiramayashi, Y., and Sugahara, K. (2002) J. Biol. Chem. 277, 31877–31886
34. Olson, S. K., Bishop, J. R., Yates, J. R., Oegema, K., and Esko, J. D. (2006) J. Cell Biol. 173, 985–994
35. Fieber, C., Baumann, P., Vallon, R., Temeer, C., Simon, J. C., Hofman, M., Angel, P., Herrlich, P., and Sleeman, J. P. (2004) J. Cell Sci. 117, 359–367
36. Sugahara, K. N., Hirata, T., Hayasaka, H., Stern, R., Murai, T., and Miyasaka, M. (2006) J. Biol. Chem. 281, 5861–5868
37. Hosono, K., Nishida, Y., Koudson, W., Knudson, C. B., Naruse, T., Suzuki, Y., and Ishiguro, N. (2007) Am. J. Pathol. 171, 274–286
38. Rolls, A., Avidan, H., Cahalon, L., Schori, H., Bakalash, S., Litvak, V., Lev, S., Lider, O., and Schwartz, M. (2004) Eur. J. Neurosci. 20, 1973–1983

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