Chondroitin sulfate is a linear polysaccharide of alternating \(\text{D-glucuronic acid and N-acetyl-D-galactosamine residues with sulfate groups at various positions of the sugars. It interacts with and regulates cytokine and growth factor signal transduction, thus influencing development, organ morphogenesis, inflammation, and infection. We found chondroitinase activity in medium conditioned by baculovirus-infected insect cells and identified a novel chondroitinase. Sequence analysis revealed that the enzyme was a truncated form of occlusion-derived virus envelope protein 66 (ODV-E66) of \textit{Autographa californica} nucleopolyhedrovirus. The enzyme was a novel chondroitin lyase with distinct substrate specificity. The enzyme was active over a wide range of pH (pH 4–9) and temperature (30–60 °C) and was unaffected by divalent metal ions. The ODV-E66 truncated protein digested chondroitin most efficiently followed by chondroitin 6-sulfate. It degraded hyaluronan to a minimal extent but did not degrade dermanatan sulfate, heparin, and N-acetyltetrapanosan. Further analysis using chemoenzymatically synthesized substrates revealed that the enzyme specifically acted on glucuronate residues in non-sulfated and chondroitin 6-sulfate structures but not in chondroitin 4-sulfate structures. These results suggest that this chondroitinase is useful for detailed structural and compositional analysis of chondroitin sulfate, preparation of specific chondroitin oligosaccharides, and study of baculovirus infection mechanisms.

Chondroitin sulfate (CS)\(^2\) is a linear polysaccharide of \((-4\text{D-glucuronic acid (GlcUA)}\) \(\beta\)1–3 \(\text{N-acetyl-D-galactosamine (GalNAc)}\) \(\beta\)1-)\(_n\), repeating disaccharide units carrying sulfate groups at various positions of the sugar residues (1, 2). CS chains are covalently attached to a core protein, in the form of proteoglycans, and are present ubiquitously in extracellular matrices and on cell surfaces. CS chains play various biological roles in development, organ morphogenesis, inflammation, and infection by interacting with cytokines and growth factors, regulating their signal transduction (3). These functions are mainly ascribed to the sulfate groups, building structural specificity and diversity into the polysaccharide framework.

Major disaccharide structures of CS are the non-sulfated unit (GlcUA-GalNAc, 0S), monosulfated unit at the C-4 position of the GalNAc residue (GlcUA-GalNAc (4-sulfate), 4S), monosulfated unit at the C-6 position of the GalNAc residue (GlcUA-GalNAc (6-sulfate), 6S), disulfated unit at the C-4 and C-6 positions of the GalNAc residue (GlcUA-GalNAc (4-, 6-sulfates), diSE), and disulfated unit at the C-2 position of GlcUA and the C-6 position of GalNAc residues (GlcUA (2-sulfate)-GalNAc (6-sulfate), diSD). During biosynthesis of CS chains, some GlcUA residues are epimerized into l-iduronic acid (IdoUA). The chain containing IdoUA residues is designated as a subfamily member of CS, dermanatan sulfate (DS).

Studies of CS/DS chains have been developed by extensive use of enzymes that specifically digest these chains. These enzymes depolymerize the substrates via a \(\beta\)-elimination reaction, which generates an unsaturated 4,5-bond on the uronic acid at the site of cleavage. Chondroitin ABC lyase I (cABC I) (4) and II (cAC II) (5) are prepared from \textit{Proteus vulgaris}. Chondroitin AC lyase I (cAC I) (4) and II (cAC II) (6) are from \textit{Flavobacterium heparinum} and \textit{Arthrobacter aurescens}, respectively. Chondroitinase B (7) is from \textit{F. heparinum}. Each enzyme exhibits a unique specificity. cABC I is an endoeliminase that depolymerizes chondroitin-4-sulfate (4S), 6-sulfate (6S), and DS to their respective unsaturated di- and tetrasaccharides. cAC I is an exoeliminase that cleaves CS and DS at the non-reducing end and digests short oligosaccharides more efficiently than cABC I. Both cAC I and cAC II depolymerize CS and 6S, but not DS. Chondroitinase B cleaves the 1-4 glycosidic bond of the DS IdoUA residue and does not cleave that of the GlcUA residue of CS and DS. These chondroitin lyases have been widely used for quantification and structural analysis of CS/DS and production of anti-CS/DS monoclonal antibodies that recognize neo-epitopes generated by these chondroitinases. In addition, they have been applied to treatment of intervertebral disc protrusion (8) and spinal cord injury (9).

In our experiments, wherein chondroitin sulfotransferases were expressed in a baculovirus system, we found chondroitinase activity in the conditioned medium of baculovirus-in-
fected insect cells. We purified the enzyme and identified it as a truncated molecule of occlusion-derived virus envelope protein 66 (ODV-E66) (10) of Autographa californica nuclear polyhedrovirus (AcMNPV). The truncated molecule has chondroitinase activity distinct from that already reported.

**EXPERIMENTAL PROCEDURES**

**Materials**—The Bac-to-Bac baculovirus expression system, Spodoptera frugiperda (sf9) insect cells, and SF-900 II serum-free medium were purchased from Invitrogen. Chemically desulfated chondroitin (CH, 99% 0S) from chondroitin sulfate from shark cartilage, CS from whale cartilage (CS-A, 77% 4S and 20% 6S), CS from shark cartilage (CS-C, 62% 4S and 24% 4S), CS from bovine trachea cartilage (CS-AC, 58% 4S and 36% 6S), CS from shark fin cartilage (CS-D, 28% 4S, 50% 6S, and 15% diSD), CS from squid cartilage (CS-E, 38% 4S, 20% 6S, and 36% diSE), DS from pig skin, hyaluronan (HA) from rooster comb, N-acetylheparosan (HPR) from Escherichia coli strain K5, heparan sulfate from pig kidney, heparin from pig intestine, and chondroitinase ABC (cABC, mixed enzyme reagent of cABCI and cABCII) were obtained from Seikagaku Corp. Chemo-enzymatically synthesized CH (sCH, 100% 0S), with an average molecular weight of 12,000, was prepared using chondroitin polymerase from *E. coli* strain K4 (K4CP), as reported previously (11–15). Chemo-enzymatically synthesized chondroitin 4-sulfate and 6-sulfate were generated by reacting sCH with chondroitinase ABC (cABC, mixed enzyme reagent of cABCI and cABCII) and chondroitin-4-sulfate and 6-sulfate were generated by reacting sCH with chondroitinase ABC (cABC, mixed enzyme reagent of cABCI and cABCII). The concentrated solution (200 μL) was applied to a column of Superdex 75 equilibrated with 50 mM Tris-HCl, pH 7.2, containing 50 mM NaCl. The fractions (0.5 ml/tube) containing chondroitinase activity were collected. The sample was precipitated by adding 100% trichloroacetic acid to a final concentration of 5%. The precipitate was washed with acetone and applied to SDS-polyacrylamide gel electrophoresis.

**Identification of the Enzyme**—The proteins in the polyacrylamide gel were detected with a silver staining kit for mass spectrometry (Wako, Japan). The protein band corresponding to chondroitinase activity was excised from the gel and digested with trypsin. The extracted solution containing digested peptides was concentrated in vacuo, desalted with Zip-Tip C18 (Millipore), and extracted with 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile containing α-cyano-4-hydroxycinnamic acid matrix solution. The extracted solution was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with an AutoFlex mass spectrometer (Bruker), and the corresponding protein was identified with the MASCOT search system (Matrix Science) (18).

The proteins in the polyacrylamide gel were transferred to a PVDF membrane (Immobilon P, Millipore) for Western blotting. The membrane was stained with Coomassie Brilliant Blue, and the protein band corresponding to chondroitinase activity was excised from the membrane. The N-terminal amino acid sequence of the protein in the membrane strips was determined with a Procise LC 492 protein sequencer (Applied Biosystems).

**Cloning and Expression of ODV-E66 Proteins—**AcMNPV baculovirus DNA was obtained from DH10Bac *E. coli* cells (Invitrogen) containing the bacmid DNA. The ODV-E66 gene was PCR-amplified using the baculovirus DNA as template and the primers, 5′-GGCTCTAGATGTCTATCGTATTGAATTGTATAG-3′ and 5′-CGCGGATCCTTACACAGATTTTCAAAAATATTGTTAAT-3′, where the XbaI and BamHI cleavage sites are underlined. The PCR product was subcloned into pBluescript KS(−) (Stratagene). The expression plasmids encoding full-length ODV-E66 and its truncated forms, starting with 24-, 67-, and 70-amino acid residues, were constructed by PCR and subcloning of the products into the PET15b expression vector (Novagen), which harbors a His tag sequence. The forward primers were 5′-GGCGGCCTATGATGTCTATCGTATTGATTATGTCTAG-3′, 5′-CCGTGCGCATGAA-TATAAAATATGGCATAAATAAAC-3′, 5′-CCGGTTGCGATATGGCTTTTCCGAAAACACCATCAAG-3′, and 5′-GGTGCCCATATGCAAAACATTTCAAGACTACAAAAC-3′, for full-length and truncated forms starting with 24, 67, and 70, respectively; Ndel cleavage sites are underlined. The reverse primer for PCR was 5′-CGCGGATCCTTACACATTTTCAAAAATATTGTTAATC-3′ for all expression plasmids; the BamHI cleavage site is underlined.

*E. coli* BL21(DE3) cells (Novagen) were transformed with the expression plasmids (pET-E66-1, -24, -67, and -70) and cultured in 2×YT medium containing 50 μg/ml ampicillin for 3 h at 37 °C. The cultures were cooled to 28 °C and then supplemented with isopropyl β-D-thiogalactopyranoside (1 mM). After 3 h of culture, the bacteria were harvested by centrifugation at 6000 × g for 10 min, resuspended in 50 mM Tris-HCl, pH 7.2, containing 50 mM NaCl and 0.2 mg/ml lysozyme, lysed by sonication on ice, and then centrifuged at 13,000 × g for 15 min at 4 °C.
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The sample was applied to a DEAE-Sepharose column equilibrated with 50 mM Tris-HCl, pH 7.2, containing 50 mM NaCl, and the expressed proteins were eluted with a linear gradient of 50–500 mM NaCl. The fractions with chondroitinase activity were collected and then applied to a nickel-Sepharose column and washed with 50 mM Tris-HCl, pH 7.2, containing 500 mM NaCl and 20 mM imidazole. His-tagged proteins were eluted with 50 mM Tris-HCl, pH 7.2, containing 500 mM NaCl and 500 mM imidazole and dialyzed against 50 mM Tris-HCl, pH 7.2 containing 150 mM NaCl.

Enzyme Activity and Kinetic Analysis—Chondroitin lyase activity was measured by absorbance at 232 nm (A_{232}) essentially as described by Yamagata et al. (4). A typical incubation mixture contained a measured quantity of enzyme protein and 100 μg of substrate in 50 μl of 50 mM phosphate buffer, pH 6.8. Control medium was a blank mixture of heat-inactivated enzyme. After incubation at 37 °C for 30 min, the reaction was stopped by heating for 1 min in a boiling water bath. The reaction mixture was then diluted with 450 μl of distilled water, and UV absorption was measured at 232 nm against the corresponding blank mixture. One unit of enzyme was defined as the activity required for the eliminative cleavage of substrate, yielding GlcUA residues per min, as calculated with a value of 5500 for its molar absorption coefficient (ε) (5). Protein concentrations were determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard.

For kinetic analysis, chondroitin lyase activities of recombinant E66 proteins (2 ng/ml) were measured with various concentrations (0.04–1.2 mM) of CH as substrate in the reaction solutions (50 μl) at 37 °C for 10 min. Kinetic parameters (K_{m} and V_{max}) were determined with double-reciprocal plots (Lineweaver-Burk plots) of chondroitin lyase activities (ν, nmol/min) and substrate concentrations (S, μmol) from the Michaelis-Menten equation. The k_{cat} was calculated by dividing V_{max} by the concentration of enzyme in the reaction. The analyses were performed in at least three experiments.

Compositional Analysis of Depolymerized Products—The digests of CH and CS (20 μg) were treated with purified E66 (67–704) protein (0.5 μg) at 37 °C for 24 h and divided into di-, tetra-, and longer saccharides by gel filtration chromatography on the Superdex peptide column (1.0 × 300 mm). The flow rate was 1.0 ml/min using 0.2 M NaCl developing buffer. The elution was determined by UV absorption at 220 nm to detect the unsaturated GlcUA and N-acetyl group of GalNAc residues. The divided fractions, except the disaccharide fraction and original substrates, were digested completely with eABC to the unsaturated disaccharides, whose compositions were measured with fluorometric post-column high performance liquid chromatography (HPLC) as described previously (19).

RESULTS

Purification and Identification of Putative Chondroitinase—In a series of experiments in which CH was sulfated with recombinant chondroitin sulfotransferases expressed with baculovirus systems, we noticed that some recombinant fractions from the conditioned medium of baculovirus-infected insect cells generated short CS chains. The conditioned medium of the baculovirus-infected sf9 cells, even without sulfotransferase cDNA, showed significant chondroitinase activity, whereas that obtained from non-infected host cells did not (data not shown), suggesting that the activity was present in the baculovirus. The solution containing CH-degrading activity yielded digestion products mainly composed of di- and tetrasaccharides that absorbed UV light at 232 nm. The prepared disaccharides showed the same elution patterns as authentic unsaturated disaccharides in the HPLC assay system. The tetrasaccharides represented molecular masses corresponding to unsaturated tetrasaccharides in MALDI-TOF MS analysis (data not shown). Thus, the enzyme expressed by baculovirus is a chondroitin lyase.

The lyase-active protein in the virus-infected cell culture medium was partially purified by sequential steps of column chromatography with DEAE-Sepharose, Q Sepharose, and Superdex 75 columns. SDS-PAGE analysis of the chondroitinase fractions of Superdex 75 showed some proteins containing a major band of 61 kDa (supplemental Fig. 1). The major band was excised from the polyacrylamide gel, digested with trypsin, and analyzed by MALDI-TOF MS and the MASCOT search system. The protein was identified as ODV-E66 of AcMNPV. In parallel, the 61-kDa band in the gel was transferred to a PVDF membrane, excised, and analyzed. Its N-terminal amino acid sequence was AFRQNNIQEL, which corresponds to amino acid residues 67–76 of ODV-E66. These results strongly suggested that ODV-E66 was responsible for the chondroitin lyase activity in the conditioned medium.

Expression of ODV-E66 Proteins and Measurement of the Enzyme Activities—ODV-E66 consists of 704 amino acids (10), and its molecular mass is calculated to be 81 kDa. The protein obtained from the baculovirus-infected insect cell-conditioned medium started with an N-terminal amino acid residue 67. Previously, a mature protein of ODV-E66 in the occlusion-derived virus (ODV) envelope fraction was reported to be a truncated form starting at amino acid residue 70 (10), and the 23 N-terminal amino acids of ODV-E66 directed viral proteins to the host nucleus (20). Therefore, we generated expression plasmids for truncated forms starting at residues 67 (E66 (67–704)) and 70 (E66 (70–704)), as well as a full-length (E66 (1–704)) and that lacking the N-terminal sequence (E66 (24–704)). They were expressed with an N-terminal His_{6} tag in a bacterial expression system. The recombinant truncated proteins E66 (24–704), E66 (67–704), and E66 (70–704) were expressed and purified by DEAE-Sepharose and nickel-Sepharose column chromatography; in contrast, the full-length protein was minimally expressed. The purified E66 (24–704), E66 (67–704), and E66 (70–704) migrated as major bands of 66, 62, and 61 kDa, respectively, on 10% SDS-polyacrylamide gel and Western blotting membrane (Fig. 1); their calculated molecular masses are 78.8, 74.3, and 73.9 kDa. E66 (67–704) and E66 (70–704) showed high chondroitin lyase activity, whereas E66 (24–704) exhibited significant but lower activity. The specific activities of E66 (24–704), E66 (67–704), and E66 (70–704) using CH as the substrate were 8.91 ± 0.18, 67.2 ± 2.1, and 61.8 ± 3.8 units/mg of protein (Table 1). Kinetic parameters of the recombinant deletion proteins were determined against CH substrate (Table 1). The kinetic
data corroborated the specific activities of the recombinant enzymes.

**Properties of the Enzyme Activity**—We examined the effect of pH on the chondroitinase activity of E66 (67–704), using various buffers including glycine-HCl (pH values of the reaction mixture were 2.9, 4.1, and 5.6), sodium acetate-acetic acid (pH 4.9, 5.9, and 6.8), potassium sodium phosphate (pH 6.3, 7.0, and 7.8), Tris-HCl (pH 7.6, 8.4, and 9.2), and CAPS-NaOH (pH 8.9, 10.1, and 10.5). The chondroitinase activity was highest at pH 7.0, 80% at pH 4 and 9, and ~30% at pH 3 and 10 (Fig. 2A).

Next, we examined the effects of divalent metal ions, chelating reagent, and sodium chloride concentration. The chondroitin lyase activity of E66 (67–704) was unaffected by 20 mM divalent metal salts (CaCl₂, MgCl₂, MnCl₂, and CoCl₂) and 20 mM EDTA when compared with reaction in 50 mM potassium sodium phosphate buffer. Activity was unaffected at 0.15 M NaCl and decreased to ~63% in 0.5 M NaCl (Fig. 2B).

We also examined the effect of reaction temperature. The activity of E66 (67–704) increased linearly at 4–37 °C and was highest at 37 °C. The activity was maintained up to 60 °C and decreased rapidly at temperatures above 65 °C (Fig. 2C). The activity of E66 (70–704) showed essentially the same patterns with variations in pH, metal ions, and temperature (data not shown).
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TABLE 1
Chondroitin lyase activities and kinetic parameters of recombinant ODV-E66 proteins

| Proteins         | Specific activity a | % b | Km c | kcat d | kcat/Km e |
|------------------|--------------------|-----|------|--------|----------|
|                  | μmol/min/mg of protein | μM  | μM−1 min−1 | μM−1 min−1 |
| E66 (24–704)     | 8.91 ± 0.18 d       | 13.3| 80.2 | 775    | 9.67      |
| E66 (67–704)     | 67.2 ± 2.1          | 100 | 421  | 6510   | 15.5      |
| E66 (70–704)     | 61.8 ± 3.8          | 91.9| 547  | 6220   | 11.4      |

a Values were obtained as described under “Experimental Procedures” using 50 ng of the proteins and 100 μg of CH in 50 μl buffer. The reaction mixtures were incubated at 37°C for 30 min.
b The relative activities are shown when the activity of E66 (67–704) is 100%.
c Kinetic parameters were obtained as described under “Experimental Procedures.”
d The reaction mixtures (50 μl) containing 50 ng of proteins and 0.04–1.2 mM CH were incubated at 37°C for 10 min.
e Data are the means ± S.D. of three independent experiments.

Substrate Specificity—Next, we investigated the substrate specificity of ODV-E66 using various glycosaminoglycans. When these substrates (100 μg) were treated with 0.05 μg of recombinant enzyme E66 (67–704) at 37°C for 30 min, the substrates were digested as summarized in Table 2. CH was the best substrate. All the CS substrates prepared from different animals were digested to lesser extents (16–30%) than CH. HA was minimally digested by ODV-E66 protein (0.16%) in a range of pH 5–8. The enzyme did not digest DS, heparin, heparan sulfate, and HPR even when the enzyme was present at 10-fold excess (0.5 μg).

We analyzed the digestion products of individual reactions using 20 μg of substrate and 0.5 μg of E66 (67–704) at 37°C for 24 h by separation on a Superdex peptide column (Fig. 3). CH was completely digested to tetra- and disaccharides (Fig. 3A), whereas the reaction products of other CS substrates revealed partial depolymerization. Both CS-A and CS-E (Fig. 3, B and F) showed stronger resistance to depolymerization by ODV-E66 than did CS-C, CS-AC, and CS-D (Fig. 3, C–E). Large proportions of long oligo- and polysaccharides remained in the reactions of CS-E (94.2%) and CS-A (78.1%) after 24 h. In contrast, 33–42% of CS-C, CS-AC, and CS-D was digested to di- or tetrasaccharides (Table 3).

We analyzed the disaccharide composition of the digestion products using a fluorescent post-column HPLC system. Interestingly, the disaccharide fractions obtained from all the CS digestions consisted mainly of 0S and 6S disaccharides, and only a small amount of 4S disaccharide was detected in the CS-C and CS-AC digests. We further analyzed the disaccharide composition of the tetrasaccharide fractions of the ODV-E66 digests by treating them with CABC. More than half the disaccharide residues of the tetrasaccharide fractions consisted of 0S and 6S, regardless of the disaccharide composition of the CS sources (Table 3). These results indicate that the enzyme preferentially digests polysaccharides having CH and C6S structures.

To characterize the substrate specificity of ODV-E66 chondroitinase activity, we used sCH, sC4S, and sC6S substrates, which had an average molecular weight of 12,000 and consisted of 100% non-sulfated, 91.9% 4-sulfated, and 93.9% 6-sulfated disaccharides, respectively. We treated these substrates with E66 (67–704) at 37°C for 24 h and separated the digests by gel filtration chromatography (Fig. 4, A–C). Most of the sCH and sC6S substrates were degraded to di- and tetrasaccharides. In contrast, sC4S was minimally digested, and both long oligosaccharides and polysaccharides remained in the reactions. The yields of di- and tetrasaccharides in the sCH and sC6S reactions were ~60 and 40%, and their disaccharide compositions were similar to the source polysaccharides (Table 4). A further reaction for an additional 24 h, by adding the same amount of the enzyme, did not alter the compositions of di- and tetrasaccharides (data not shown). Therefore, they were limited digests. The ratios of the di- and tetrasaccharides digested from sC4S were 4.8% and 2.6%, respectively, and 92.5% of the reaction remained as polysaccharides. The separated di- and tetrasaccharide fractions from sC4S contained large proportions of non-sulfated units, distinct from the source polysaccharide, whereas
the long saccharide fraction contained a proportion of the 4-sulfated unit, similar to the source polysaccharide (92.4% versus 91.9%, Table 4).

**DISCUSSION**

We found CS-degrading activity in a baculovirus expression system and identified the enzyme as a truncated form of ODV-E66, one of the envelope proteins of AcMNPV baculovirus. ODV-E66 truncated protein is a novel chondroitin lyase with distinct substrate specificity. It selectively cleaves 0S and 6S units of CS chains. This is the first report of a CS-degrading enzyme in viruses. The enzyme is a new chondroitinase with unique substrate specificity and utility in CS analysis.

To date, various chondroitinases (4–7) and hyaluronidases (21, 22) have been identified in many types of bacteria. They have unique substrate specificities but often share substrates. For example, cABCII and cABCIII degrade C4S, C6S, and DS. cACI and cACII degrade C4S and C6S, but not DS. cABCII, cACI, and cACII degrade both CS and HA, to a greater or less extent. There are two opposing studies on whether cABCII digests HA (5, 23). HA lyase (21) from *Streptomyces hyalurolyticus* does not digest CS/DS, whereas that from *Streptococcus* sp. digests CH to a lesser extent than HA (22). When compared with bacteria chondroitinases, ODV-E66 showed narrow substrate specificity with regard to CS structure. It cleaves both 0S and 6S units of CS chains but does not cleave the other units (4S, diSD, and diSE). It degrades HA with quite low activity when compared with CH and does not degrade DS. ODV-E66 is an endoeliminase as various oligosaccharides were generated as intermediates from CS substrates containing undigestible disaccharide units in the reaction.

**TABLE 2**

| Substrate Source | Relative activity $\%$ |
|------------------|------------------------|
| CH               | 100 ± 3.1*             |
| CS-A             | 23.8 ± 0.5             |
| CS-C             | 28.0 ± 0.6             |
| CS-AC            | 29.3 ± 0.5             |
| CS-D             | 22.5 ± 0.4             |
| CS-E             | 16.1 ± 0.3             |
| DS               | <0.01                  |
| HA               | 0.16 ± 0.04            |
| Heparin          | <0.01                  |
| Heparan sulfate  | <0.01                  |
| HPR              | <0.01                  |
| Pig skin         | 23.8 ± 0.5             |
| Whale cartilage  | 28.0 ± 0.6             |
| Shark cartilage  | 29.3 ± 0.5             |
| Shark fin cartilage | 22.5 ± 0.4         |
| Squid cartilage  | 16.1 ± 0.3             |
| Pig intestine    | <0.01                  |
| Pig kidney       | <0.01                  |
| Pig skin         | 23.8 ± 0.5             |
| Whale cartilage  | 28.0 ± 0.6             |
| Shark cartilage  | 29.3 ± 0.5             |
| Shark fin cartilage | 22.5 ± 0.4         |
| Squid cartilage  | 16.1 ± 0.3             |
| Pig intestine    | <0.01                  |
| Pig kidney       | <0.01                  |
| Pig skin         | 23.8 ± 0.5             |
| Whale cartilage  | 28.0 ± 0.6             |
| Shark cartilage  | 29.3 ± 0.5             |
| Shark fin cartilage | 22.5 ± 0.4         |
| Squid cartilage  | 16.1 ± 0.3             |
| Pig intestine    | <0.01                  |
| Pig kidney       | <0.01                  |

*The enzyme activities of E66 (67–704) were measured with various glycosaminoglycans (100 μg). The reaction mixtures (50 μl) containing depolymerized substrates (CH and groups of CS) were incubated with 50 ng of E66 (67–704) at 37 °C for 30 min. For substrates DS, HA, heparin, heparan sulfate, and HPR, 500 ng of E66 (67–704) protein was used.

* Data are means ± S.D. of three independent experiments.

**FIGURE 3.** Gel filtration chromatography of the E66 (67–704) CS digestion products. A–F, CH (A), CS-A (B), CS-C (C), CS-AC (D), CS-D (E), and CS-E (F) were treated with E66 (67–704) proteins at 37 °C for 24 h as described under “Experimental Procedures.” The digests (solid lines) and the original substrates (broken lines) were separated on a Superdex peptide column. The elution was monitored by UV absorption at 220 nm. $V_0$ and $V_t$ indicate the position of void and total volume of the column, respectively. The peaks numbered 2 and 4 indicate di- and tetrasaccharides.
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TABLE 3
Disaccharide compositions of various chondroitin sulfates and the digested fractions and molar ratios of the disaccharide units of the fractions

The substrates were treated with E66 (67–704) at 37 °C for 24 h as described under “Experimental Procedures” (Fig. 3). The abbreviations used are: di, disaccharide fraction; tetra, tetrasaccharide fraction; long, longer saccharides than tetrasaccharide fraction; origin, CS substrate before digestion; ND, not detected.

| Substrate | Fraction | 0S | 4S | 6S | diSE | diSD | Molar ratioa |
|-----------|---------|----|----|----|------|------|-------------|
| CH        | di      | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 38.8 |
|           | tetra   | 99.0 | 0.0 | 1.0 | 0.0 | 0.0 | 61.2 |
|           | long    | ND   | ND | ND | ND | ND | 0.0 |
|           | origin  | 99.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 |
| CS-A      | di      | 14.4 | 85.6 | 0.0 | 0.0 | 0.0 | 11.2 |
|           | tetra   | 8.1 | 34.1 | 57.9 | 0.0 | 0.0 | 10.7 |
|           | long    | 2.6 | 81.9 | 13.8 | 0.2 | 1.4 | 78.1 |
|           | origin  | 0.8 | 77.3 | 20.2 | 0.3 | 1.4 | 78.1 |
| CS-C      | di      | 1.7 | 98.0 | 0.0 | 0.0 | 0.0 | 32.4 |
|           | tetra   | 0.2 | 9.9 | 89.0 | 0.9 | 0.0 | 14.5 |
|           | long    | 2.4 | 34.3 | 45.6 | 3.1 | 14.6 | 53.1 |
|           | origin  | 3.4 | 23.5 | 62.0 | 1.7 | 9.4 | 26.1 |
| CS-AC     | di      | 1.9 | 96.4 | 0.0 | 0.0 | 0.0 | 13.7 |
|           | tetra   | 10.8 | 24.0 | 65.1 | 0.0 | 0.0 | 19.6 |
|           | long    | 2.5 | 72.0 | 24.6 | 0.5 | 0.4 | 66.7 |
|           | origin  | 5.7 | 57.5 | 36.1 | 0.4 | 0.3 | 26.1 |
| CS-D      | di      | 1.8 | 98.0 | 0.0 | 0.0 | 0.0 | 26.1 |
|           | tetra   | 1.2 | 10.9 | 85.7 | 2.2 | 0.0 | 12.0 |
|           | long    | 2.5 | 35.3 | 40.5 | 4.0 | 18.4 | 62.0 |
|           | origin  | 3.5 | 28.1 | 50.3 | 3.0 | 15.2 | 78.1 |
| CS-E      | di      | 64.8 | 0.0 | 35.2 | 0.0 | 0.0 | 1.5 |
|           | tetra   | 9.8 | 27.9 | 42.3 | 20.1 | 0.0 | 4.3 |
|           | long    | 4.6 | 34.7 | 21.2 | 39.5 | 0.0 | 94.2 |
|           | origin  | 5.1 | 38.4 | 20.4 | 36.1 | 0.0 | 0.0 |

a Mole percent of compositions of the disaccharide units.
b Molar ratio as disaccharide units.

cABCI, cABCI, cACI, and chondroitinase B are most active around pH 8, whereas cACII and HA lyases act under acidic conditions (pH 5–6). In contrast, ODV-E66 exhibited high activity over a wide pH range. Optimum temperatures for cABCI, cABCI, cACI, and chondroitinase B activity are 37, 37, 40, 50, and 30 °C, and their activity decreases immediately at higher temperatures. In contrast, ODV-E66 exhibited high activity over a wide range of temperatures. These observations suggest that ODV-E66 maintains a stable structure under different conditions.

The enzyme activities of both chondroitinase B from *F. hepaticum* (24) and cABC from *Bacteroides thetaiotaomicron* (25) are affected by divalent metal ions such as Ca$^{2+}$ ion and are inhibited by chelators such as EDTA and EGTA. Ca$^{2+}$ and Mg$^{2+}$ ions preferentially increase the activity of cABCI toward DS versus CS (26). These chondroitinases have a Ca$^{2+}$ binding region at the active site. In contrast, the chondroitinase activity of ODV-E66 was unaffected by divalent ions and chelating reagent, suggesting that the structure of its catalytic site may differ from those of bacterial chondroitinases. Structural analysis of the ODV-E66 catalytic sites remains to be performed to characterize its mechanism of action.

Specific activity and kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of E66 (67–704) for CH were 67.2 μmol/min/mg of protein, 421 μM, 6510 min$^{-1}$, and 15.5 min$^{-1}$ μM$^{-1}$, respectively. Those of cABCI (26, 27) were reported as 170–230 μmol/min/mg of protein, 1.2–2.4 μM, 2200–3700 min$^{-1}$, and 3100–9100 min$^{-1}$ μM$^{-1}$. Those of cABCI (23) were 29–32 μmol/min/mg of protein, 9.8 μM, 1300 min$^{-1}$, and 132 min$^{-1}$ μM$^{-1}$. Specific activities of other bacterial lyases were 40–320 μmol/min/mg of protein. The high $K_m$ of E66 (67–704) indicates its low substrate binding affinity in comparison with bacterial chondroitinases, although the order of the specific activity of ODV-E66 appears similar.

Full-length ODV-E66 contains a 23-amino acid signal sequence at the N terminus, a polysaccharide lyase family sequence (amino acids 83–291), and a baculovirus E66 superfamily sequence at the C terminus (residues 316–704) (NCBI Conserved Domain Search). Mature protein isolated from the conditioned medium of baculovirus-infected cells was a truncated form starting at amino acid 67. A truncated form starting at amino acid 70 was previously reported as a mature protein of ODV-E66 (10). Recombinant proteins E66 (67–704) and E66 (70–704) showed high chondroitin lyase activity, whereas E66 (24–704), which lacked the N-terminal signal sequence, showed significantly lower activity than the mature proteins. Thus, it is likely that ODV-E66 is processed by certain proteases that remove the N-terminal fragments of amino acids 66 and 69 to achieve high CH/CS-degrading activity.

Insects such as fruit fly (*Drosophila*) and mosquito (*Anopheles*) have low sulfated CS proteoglycans. CS proteoglycans have been detected in *Drosophila* ovaries, embryos, larvae, and adults (28, 29) and *Anopheles* salivary glands, midguts, and ovaries (30). By histological analysis, the CS proteoglycans were found in the apical microvilli of the mosquito midgut (31). CS of fruit fly and mosquito is composed mainly of 0S disaccharide units and only a small amount of 4S disaccharide units. *Drosophila* larvae in the L3 stage contain significant amounts of 6S (32). Therefore, ODV-E66, which degrades CH and CS, may be beneficial for CS digestion in insects.
and moves to the midgut. Lepidopteran larvae generate the peritrophic matrix in the luminal side of the midgut, which protects the mucosa from virulence (34). The peritrophic matrix of lepidopteran larvae may contain CS glycosaminoglycans as in other insects.

ODV-E66 may be released with other envelope proteins from the midgut occlusion body with the high pH and proteinases secreted from the midgut epithelia. The truncated ODV-E66 that acquires CH-degrading activity may digest the CS barrier of the peritrophic matrix of the host midgut and facilitate viral infection to the epithelial cells. Vigdorovich et al. (35) previously reported that ODV-E66 had hyaluronidase activity, which was confirmed in this study. However, its activity on HA was ~600 times lower than its CH-degrading activity. It is not necessary to have HA-degrading enzymes for baculovirus pathogenesis of insects because insects have neither HA nor HA synthase genes (36).

The N-terminal 23 amino acids of ODV-E66 were reported to traffic viral proteins to the inner nuclear membrane (20, 37). Baculovirus envelope proteins are transported to the inner nuclear membrane with the N-terminal sequence of ODV-E66. The occlusion bodies are formed in the nucleus and then released to the outside environment. At that time, the active ODV-E66 protein may be expressed, degrading the extracellular matrix containing CS and facilitating the disintegration of the infected host.

ODV-E66 will be included as a new member of the chondroitinases with unique substrate specificity and utility in CS analysis. The enzyme may be useful to prepare highly sulfated oligosaccharides, including diSD and diSE units, which are known to possess various biological activities. Moreover, ODV-E66 may facilitate the virus infection to insect host and be useful for study of baculovirus infection mechanism. Recent studies (8, 9) have revealed that chondroitinases may be useful in treatment of spinal cord injuries and intervertebral disc herniation. As ODV-E66 digests CS over a wide range of temperature and pH, it may be another candidate for such medical applications.

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