Purification, Characterization, and Molecular Cloning of a Novel Keratan Sulfate Hydrolase, Endo-β-N-acetylgalcosaminidase, from Bacillus circulans*

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Keratan sulfate (KS) is degraded by various enzymes including endo-β-galactosidase, keratanase, and keratanase II, which are used for the structural analysis of KS. We purified a novel KS hydrolase, endo-β-N-acetylgalcosaminidase, from the cell pellet and conditioned medium of Bacillus circulans, by sequential chromatography using DE52 and phenyl-Sepharose columns with ~63- and 180-fold purity and 58 and 12.5% recovery, respectively. Like keratanase II of Bacillus sp. Ks36, the enzyme, designated Bc keratanase II, hydrolyzed KS between the 4GlcNAcβ1–3Galα1 structure (endo-β-N-acetylgalcosaminidase), but not hyaluronan, heparan sulfate, heparin, and chondroitin sulfate C, demonstrating a strict specificity to KS. The enzyme digested shark cartilage KS to disaccharides and tetrasaccharides and bovine cornea KS to hexasaccharides, indicating that it prefers highly sulfated KS. Distinct from keratanase II of strain Ks36, the enzyme digested shark cartilage KS at an optimal temperature of 55 °C. Based on partial peptide sequencing of the enzyme, we molecularly cloned the gene, which encodes a protein with a predicted molecular mass of ~200 kDa. From the deduced protein sequence, Bc keratanase II contained a domain at the C terminus, homologous to the S-layer-like domain of pullulanase from Thermoanaerobacterium thermosulfurigenes and endoxylanase from Thermoanaerobacterium saccharolyticum, and a carbohydrate-binding domain, which may serve to specifically recognize KS chains. A full-length recombinant enzyme showed keratanase II activity. These results may prove useful for the structural analysis of KS toward achieving an understanding of its function.

Keratan sulfate (KS)† is a glycosaminoglycan (GAG) whose main structure comprises β1–3 repeats of the galactosyl β1–4-GlcNAC disaccharide unit and is classified into three types based on linkage structure as follows: KS-I, KS-II, and KS-III (1). KS-I is N-linked at the reducing end of GlcNAC to Asn residues in proteins. This type of KS is a major component of cornea and contributes to its transparency. KS-II is mainly localized in cartilage and is O-linked at the reducing end of GlcNAC to Ser/Thr in proteins, a link susceptible to alkaline treatment, compared with the N-link of KS-I. KS-III is localized in the brain and is O-linked to Ser/Thr via mannose. In addition to the linkage region, KS chains differ in sulfate content and the proportions of minor sugar components including sialic acid, fucose, and N-acetylgalactosamine. Thus, KS represents a variety of forms in tissues.

Recent studies have revealed various in vivo functions of KS chains in embryogenesis, cancer invasion, immune responses, and cartilage metabolism. The KS chain is expressed in various tissues in the embryo and regulates organogenesis by affecting filtration and differentiation in the kidney (2), migration of primary germ cells in the gonads or of neural crest cells in the heart and gut (3), and axonal guidance in the gut and skin (2). A sialylated KS proteoglycan serves as a specific marker for human embryonal carcinoma, which may regulate the biological behavior of the tumor (4). 6-Sulfo-N-acetylgalactosaminic acid has been identified as an epitope of dendritic cell-specific monoclones, M-DC8, DD1, and DD2, suggesting an important role for this saccharide structure in T cell stimulation by dendritic cells (5). KS is known to be important for aggrecan degradation by aggrecanase-1, a key enzyme in cartilage metabolism (6). Many KS-containing proteoglycans, including lumican, keratan, mimcan, fibromodulin, and phosphacan, have been identified and their in vivo roles studied by using knockout mice (7, 8). As these proteoglycans function not only via their core protein but also via their GAG chains, detailed characterization of KS chains is necessary to define the structure for their specific functions.

Any fingerprinting method for analyzing the structure of KS requires both fragmentation usually with KS-degrading enzymes and subsequent separation. At present, the various KS-degrading enzymes are classified into three groups based on their cleavage sites and substrate specificity. The first are the endo-β-galactosidases identified in Escherichia freundii (9), Coccobacterium sp. (10), Flavobacterium keratolyticus (11), and Bacteroides fragilis (12), which hydrolyze the internal non-sulfated galactosidic bonds of KS. The second is the keratanase identified in Pseudomonas sp.IFO-13309 (13). This enzyme also cleaves internal β1–4 galactosidic linkages in KS but differs in substrate specificity from the endo-β-galactosidase. It requires at least one N-acetylgalactosamine 6-sulfate residue and does not hydrolyze the desulfated KS polymer. The third group is the keratanase II identified in Bacillus sp. Ks36, endo-β-N-acetylgalactosaminidase (14), which cleaves N-acetyl-
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Shark cartilage KS was digested with Bacillus sp. keratanase II. Individual oligosaccharides were purified by a Bio-Gel P-2 and a Muromac anion exchanger column chromatography and characterized using NMR and mass spectrometry, as described under "Experimental Procedures."

Table I: Major oligosaccharides produced by keratanase II of Bacillus sp. Ks36 digestion of shark cartilage KS

| Oligosaccharide structure | Mw | Retention time of GPC-HPLC | Migration time of CE |
|--------------------------|----|-----------------------------|---------------------|
| Gal(1)-4GlcNAc(6S)       | 543.47 | 43.46                      | 19.69 |
| Gal(1)-4GlcNAc(6S)       | 1068.93 | 41.18                      | 23.01 |
| NeuAcα2-3Gal(1)-4GlcNAc(6S) | 1280.13 | 40.85                      | 17.69 |
| Gal(1)-4GlcNAc(6S)       | 383.35 | 46.67                      | 8.50 |

Preparation of Specifically 6-O-Desulfated KS and KS Disaccharides—Oligosaccharide purification was performed according to the method of Kariya et al. (17). Five hundred milligrams of shark cartilage KS or KS disaccharide pyridinium salt was dissolved in 5 ml of N-methyl-N-(trimethylsilyl)-trifluoroacetamide and 50 ml of pyridine. For the preparation of desulfated KS, the mixture was incubated at 110 °C for 2 h. After cooling down on ice, an equal volume of water was added to the mixture. The sample was then evaporated, dissolved in water, dialyzed against water, and lyophilized. For preparation of desulfated KS disaccharide, the mixture was heated at 80 °C for 2 h, and an equal volume of 20% methanol was added to the reaction mixture, evaporated, and dissolved in water. The solution was then applied to a charcoal-Celite column to remove salts. After the column had been washed with 5 volumes of water, the desulfated KS disaccharide was eluted with 30% methanol, evaporated, and lyophilized.

Culture of B. circulans—B. circulans Ks7202 was maintained on brain heart infusion slant medium (20% calf brain infusion, 25% beef heart infusion, 1% proteose peptone, 0.5% NaCl, 0.25% Na HPO4, and 1.5% agar, pH 7.2) supplemented with 0.2% shark cartilage KS. After inoculation into a 500-ml flask containing 100 ml of seed medium (0.75% peptone, 0.5% Brewery’s yeast extract, 0.5% KH2PO4, 0.02% MgSO4·7H2O, 0.1% NaCl, and 0.2% shark cartilage KS, pH 7.0), the bacteria were cultured at 37 °C for 14 h in a shaker at 120 rpm. Then 90 ml of the culture was transferred into a 5-liter fermentor containing 3 liters of medium P (1.0% peptone, 0.5% Brewery’s yeast extract, 0.25% fish extract, 0.5% KH2PO4, 0.02% MgSO4·7H2O, 0.1% NaCl, 150 ppm Ade2, and 150 ppm MgCl2·6H2O) and 0.5% shark cartilage KS (pH 7.5) and further cultured at 37 °C and 200 rpm with an air flow for 8 h.

Keratanase Assay—The keratanase assay was carried out according to the method of Park and Johnson (18). The enzymatic reaction was performed in 200 μl of 100 mM acetate buffer, pH 6.0, containing 100 μg of KS. After incubating for 10 min at 37 °C, the reaction was stopped by adding 200 μl of the carbonate/KCN solution (50 mM Na2CO3 and 10 mM KCN) and 200 μl of K2Fe(CN)6 solution (1.8 mM) and boiled for 10 min. After cooling down to room temperature, the sample was mixed with 1 ml of Alum solution (3.1 mM Fe(NH4)2(SO4)2·12H2O, 0.05 mM H2SO4, and 0.1% SDS), mixed, and incubated for 15 min at room temperature. Then the amount of reducing sugar was determined from the increase in absorbance at 680 nm. A heat-inactivated sample was used as a control.

One unit of the enzymatic activity was defined as the amount of the enzyme that released 1 μmol of reducing sugars (as galactose) per min under the assay conditions.

Purification and Partial Peptide Sequencing of Be Keratanase II—B. circulans keratanase II (Bc keratanase II) was purified from bacterial pellets or culture media of cells grown in the presence of shark cartilage KS as follows. All isolation and purification procedures were carried out at 4 °C. Thirty grams of frozen bacteria obtained from 3 liters of culture was resuspended in 150 ml of phosphate-buffered saline and treated with both lysozyme (100 μg/ml) and DNase I (26 μg/ml) for 30 min at 37 °C. After centrifugation, the supernatant was extracted twice. The stepwise addition of ammonium sulfate, the KS-degrading activity was recovered in the fraction between 35 and 60% saturation. The sample fraction precipitated at 60% saturation was dissolved in 10 ml of 10 mM Tris-HCl, pH 7.2, dialyzed against the same buffer, and applied to a DE52 column (2.4 × 14 cm, Whatman) equilibrated with the same buffer.

The enzyme fraction was eluted using a gradient of up to 0.3 M NaCl. After the addition of NaCl to a final concentration of 4 M, the enzyme fraction was applied to a phenyl-Sepharose CL-4B column (1.5 × 14 cm, Amersham Biosciences) equilibrated with 10 mM Tris-HCl, pH 7.2, and 4 M NaCl. The enzyme fraction was eluted using a decreasing gradient of NaCl concentrations in the fraction from 2.3 to 2.1 M NaCl.

By using 20 liters of conditioned medium kept at 4 °C for 24 h, the protein was precipitated through 70% saturation of ammonium sulfate. The precipitate was resuspended in 10 ml Tris-HCl, pH 7.5, and the solution was subjected to stepwise precipitation with ammonium sulfate. The enzyme was precipitated with a 0.35–0.55 saturation. The enzyme fraction was applied to a DE52 column (5.2 × 24 cm) equili-
brated with 10 mM Tris-HCl, pH 7.5, and was eluted using a gradient of NaCl to 0.5 M. The protein was precipitated with 0.5 M saturation of ammonium sulfate, resuspended in 10 mM Tris-HCl, pH 7.5, and applied to a Sephacryl S-300 column (2.2 × 101 cm). The fraction containing enzyme activity was again applied to a DE52 column (5.2 × 10 cm), similarly eluted, and then concentrated using a Centricon ultrafiltration spin column. The concentrated enzyme fraction was again applied to a Sephacryl S-300 column, and the enzyme fraction was applied to a phenyl-Sepharose column (1.6 × 15 cm) and eluted as described above.

**Capillary Electrophoretic Analysis of KS Oligosaccharides**—Capillary electrophoretic analysis of KS oligosaccharides was performed by the method of Yoshida. The experiments were performed on a CE system PACE 2100 (Beckman Instruments) equipped with a fixed wavelength ultraviolet detector (UV at 214 nm). The CE system was operated in normal polarity by applying the sample at the cathode. The running buffer was 50 mM sodium tetraborate, pH 9.0. The sample was separated and analyzed using a fused silica capillary tube (75 μm × 60 cm, Beckman Instruments). Each experiment was performed at a constant voltage of 15 kV. The system operation and data handling were fully controlled, and chromatograms were integrated using System Gold chromatography-automated software (version 4) from Beckman Instruments running on a Compaq personal computer. The migration times of Gal(6S)β1–4GlcNAc(6S), Gal(6S)β1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S), and NeuAco2–3Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S) are 19.7, 23.0, and 17.7 min, respectively (Table I).

**HPLC Analysis of KS Oligosaccharides and Neutral Sugars**—The average molecular weight and yield of KS oligosaccharides were estimated by gel permeation chromatography-high performance liquid chromatography (GPC-HPLC) with serially combined columns of Tosk-gel PWXL-4000, PWXL-3000, and PWXL-2500 under a constant flow (0.6 ml/min) of 200 mM NaCl at 40 °C. Ten mg of KS was digested with 10 milliunits of the enzyme in 1 ml acetate buffer, pH 6.0, under 37 °C for 24 h. After centrifugation at 6,000 × g and 4 °C for 5 min, aliquots of the supernatant were used as the samples. The injection volume was 5 μl (19). Detection was performed with a refractive index. The retention times of Gal(6S)β1–4GlcNAc(6S), Gal(6S)β1–4GlcNAc(6S)β1–3Gal(6S)β1–4GlcNAc(6S), and NeuAco2–3Galβ1–4GlcNAc(6S)β1–3Galβ1–4GlcNAc(6S) are indicated in Table I.

The amount of N-acetylated amino sugars was determined by HPLC with a Shodex NH2-P50 column (4.6 × 250 mm; Showa Denko, Tokyo, Japan) under a constant flow (0.6 ml/min) of 75% acetonitrile solution at 40 °C. Injection was carried out at 5 μl. The eluate was monitored from the absorbance at 214 nm (N-acetyl group). The retention times of N-acetylglucosamine and N-acetyllactosamine were 10.2 and 15.2 min, respectively (Fig. 6).

**Determination of Amino Acid Sequences**—Purified enzyme was subjected to SDS-PAGE using a 4% gel. After staining with Coomassie Brilliant Blue, a homogeneous protein in the gel was excised, extracted using 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 0.5% SDS, and digested with lysyl endopeptidase (0.4 μg). The digest was applied to a reverse phase column (ODS-50A, 2 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. The resulting peptides were separated with a linear gradient of 70% acetonitrile and 0.08% trifluoroacetic acid at a flow rate of 180 μl/min. Amino acid sequences of the resulting peptides were determined with a protein sequencer (PE Applied Biosystems, model 473A). The PCR product, which corresponded to a partial genomic sequence of the enzyme gene, was subcloned into a pCR4-TOPO (Invitrogen). The 5′- and 3′-ends of the DNA were extended by inverse PCR. Appropriate restriction sites, BglII, PsI, and HindIII, were chosen for inverse PCR, using genomic Southern blot analysis with the PCR product as a probe. Inverse PCR was performed using the sense primer 5′-GATCTTGAGGCGCAAGTGTCGGGGCGCGATGAATC-3′ and antisense primer 5′-TGTAAAGGCGCCTGaCAGCTGCTGTATGTTAGG-3′ and the self-ligate of the BglII-digested genomic DNA as the template with a program of denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 min. Inverse PCR was performed using the sense primer 5′-AACGTACTGGTGATCACAATGAAGA-3′ and antisense primer 5′-AACTTTGCCATAAAGCTGATCT-3′ and the self-ligate of the PsI-digested genomic DNA as the template with the same program. Inverse PCR was performed using the sense primer 5′-CAATTCGAGCGCCAAAGCGCGGATC-3′ and antisense primer 5′-GATGTAGTATGCGAATTTAAATCACCT-3′ and the self-ligate of the HindIII-digested genomic DNA as the template. The PCR products were subcloned into pCR4-TOPO. DNA sequencing was performed using the dyeoxy chain termination method, using an ABI Prism 310 DNA sequence. Conserved domain search was performed using Pfam HMM (pfam.wustl.edu/hmmsearch.shtml) and NCBI conserved domain search (www.ncbi.nlm.nih.gov/BLAST).

**Expression of Recombinant Bc Keratanase II and Its Domains**—A DNA fragment that encodes a full-length protein enzyme was amplified by PCR using as primers 5′-ATGGATCCCTTCCATCTGGCAAGAT-3′ and 5′-GCCAATTCGCCCTTAAACAGCATGTTAAA-3′ and genomic DNA of the bacteria as template and ligated into the expres-
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RESULTS

Purification and Characterization of Bc Keratanase II—
B. circulans keratanase II was purified from either a bacterial pellet or conditioned medium. A representative purification profile obtained from the pellet is summarized in Table II. From the pellet, the enzyme was purified by 63-fold to homogeneity with 58% recovery by a two-step chromatography using DE52 and phenyl-Sepharose. Typical chromatograms of DE52 and phenyl-Sepharose are shown in Fig. 1. Digestion of the bacterial suspension with lysozyme resulted in a higher extraction efficiency than sonication or repeated freeze-thawing. Furthermore, treatment with DNase I achieved a 2.4-fold extraction efficiency (data not shown). From 30 g of bacterial pellet, 80 units with a specific activity of 1.27 units/mg was obtained. Similarly, from the conditioned medium, the enzyme was purified by 180-fold with 12.5% recovery by a five-step column chromatography using DE52, Sephacyr S-300, DE52, Sephacryl S-300, and phenyl-Sepharose. From 20 liters of conditioned medium, 29 units of the enzyme when tested as chloride salts at 1 mM. SH inhibitors such as p-chloromercuribenzoic acid and chelating agents such as EDTA showed no inhibitory effect (data not shown).

Next, we characterized the enzyme by using shark cartilage KS as a substrate. The enzyme was stable at temperatures up to 45 °C and retained 65% activity at 50 °C after incubating for 1 h in 100 mM acetate buffer, pH 6.0 (Fig. 3A). The optimal temperature for the enzyme reaction was 55 °C. The enzyme exhibited 80 and 79% activity at 50 and 60 °C, respectively (Fig. 3B). The pH stability profile of the keratanase II disclosed that the enzyme is stable at pH 6.0–7.0 (Fig. 3C). As shown in Fig. 3D, the enzyme activity was maximal at pH 5.0 in the same buffer. Effects of metal ions on the activity were examined under the keratanase assay conditions. Na⁺, K⁺, Mn²⁺, Co²⁺, Ba²⁺, Ca²⁺, and Zn²⁺ had no significant effect on the enzyme when tested as chloride salts at 1 mM. SH inhibitors such as N-ethylmaleimide, p-chloromercuribenzoic acid, and chelating agents such as EDTA showed no inhibitory effect (data not shown).

Then we examined the substrate specificity of this enzyme by using various GAG chains including bovine cornea KS and shark cartilage KS. Although both KS chains were digested, cartilage KS was hydrolyzed 1.7-fold that of cornea KS. In contrast, all the other GAG chains including hyaluronic acid, chondroitin sulfate, heparan sulfate, and heparin were resistant (Table III), demonstrating a specificity to KS chains. Interestingly, no activity was observed toward de-sulfated KS chains, indicating an absolute requirement of the presence of a sulfated residue for digestion.

Next, we determined the kinetics of the enzyme (Fig. 3E). The keratanase assay was performed at various concentrations of cornea KS and cartilage KS at 37 °C, pH 6.0. The $K_m$ and $V_{max}$ values were calculated from Lineweaver-Burk plots, suggesting that most cartilage KS was decomposed to KS disaccharides and tetrasaccharides. The $K_m$ for cornea KS and cartilage

![Fig. 2. SDS-PAGE of Bc keratanase II.](image)

![Fig. 3. General properties of Bc keratanase II.](image)
TABLE II
Substrate specificity of Bc keratanase II

The hydrolytic activities for commercial available glycosaminoglycans were determined by the method described under "Experimental Procedures." In each reaction mixture (200 µl), 0.2 milliunits of Bc keratanase II was added. The activity for each glycosaminoglycan was shown at the relative value of the activity for cartilage KS.

| Substrate         | Relative activity |
|-------------------|------------------|
| Cartilage KS      | 100              |
| Cornea KS         | 57.5             |
| Desulfated KS     | 0                |
| Hyaluronic acid   | 0                |
| Chondroitin sulfate C | 0          |
| Heparan sulfate   | 0                |
| Heparin           | 0                |

KS was 143 and 54 µg/ml, respectively, and the V\text{max} was 39.1 and 57.7 µmol/min/mg protein, respectively.

Analysis of KS Digests—We analyzed digests of shark cartilage KS and bovine cornea KS (2 mg/ml) by this enzyme, using a GPC-HPLC. Most of the cartilage KS was digested to KS disaccharide (57.5%) and tetrasaccharide (34.1%), whereas KS oligosaccharides over pentasaccharides (27.9%) remained in the cornea KS digest (Fig. 4). Monosaccharides such as GlcNAc(6S) and Gal(6S) were not detected in both digests. Similar digestion profiles were obtained when these KS samples were digested with keratanase II of Bacillus sp. Ks36 (endo-β-N-acetylgalactosaminidase) (data not shown). These results indicate that this enzyme is an endoglycosidase.

Next we determined the cleavage site of the enzyme. As the CE profiles of the digests by the enzyme agreed well with those by keratanase II of Bacillus sp. Ks36, we compared the structure of the major disaccharide fraction of shark cartilage KS digests by the purified enzyme (peak 2 in Fig. 4) and its 5-O-sulfate-specific desulfated form with various KS oligosaccharides, using CE and HPLC. In the CE analysis, the migration time of the KS disaccharide (Fig. 5B) and desulfated disaccharide (Fig. 4) was 19.7 and 8.5 min, suggesting that these products were (Gal(6S) peak 2) and (Gal(6S) peak 1) in the molecular standard profile (A) and that the enzyme was an endo-β-N-acetylgalactosaminidase, keratanase II, according to its definition. Therefore, we designate this enzyme as Bc keratanase II.

Molecular Cloning and Expression of the Keratanase II Gene—Purified enzyme excised from a SDS-PAGE gel was digested with lysyl endopeptidase, and the digests were purified using a HPLC column, followed by peptide sequencing. The
sequences of three tryptic peptides, GFLTPDLANVQNLNNIGF, FLGQPALIPTIVDAYWTAYPDG, and VEQGPADPVTEPTLLRG, were obtained and designated as peptides 1–3, respectively. Based on these peptide sequences, degenerative oligonucleotide primers were designed as described under “Experimental Procedures.”

PCR using genomic DNA of B. circulans as a template and a set of degenerative oligonucleotide primers (ATIGTIGGIATIARIGCIGGYTG and GCIGAYCCICGCIGTIACIGARCC) yielded a DNA of ∼2.5 kb. By using restriction enzyme sites, inverse PCR was performed to obtain the 5’ and 3’ sequences. The composite DNA encoded an open reading frame of 5811 bp corresponding to a protein of 1936 amino acids. Instead of a typical Shine-Dalgarno sequence of AG-GAGG, the sequence TAAAGG (nucleotides 231–236) was found upstream of the putative translational initiation site. From the deduced amino acid sequence, the molecular weight and the isoelectric point (pI) of the enzyme were calculated to be 213,333 and 4.92. A cluster of hydrophobic amino acids (Fig. 7A, 1–34), presumably a signal sequence, was located between the putative translational initiation site and the N-terminal sequence of the purified enzyme, SIRQDPTTGN.

We looked for conserved domains among published glycosidase domains using Pfam HMM search and NCBI conserved domain search, and we found two domains with homology to certain domains. A region at the C terminus of the enzyme contained three stretches (amino acids 1760–1802, 1819–1861, and 1884–1927) of sequence homologous to the SLH domain of pullulanase from Thermoaerobacterium thermosulfurigenes and endoxylanase from Thermoaerobacterium saccharolyticum. A weak homology to the consensus sequence of carbohydrate-binding domain (CBM49) was found in the region of amino acids 307–457 of the enzyme, which is found in F. keratolyticus. No other homologous domains were identified in the coding sequence of Bc keratanase II.

Next, we expressed a full-length enzyme using a pET-22b bacterial expression system and confirmed its activity. A soluble fraction of the cell extract digested KS with an activity of 83 milliunits/mg protein, 4-fold the activity of the native enzyme expressed by B. circulans. When the KS digests were analyzed using GPC-HPLC, identical patterns were obtained as digests with the native enzyme (Fig. 7B), indicating that the recombinant protein actually has the keratanase II activity. When a recombinant enzyme lacking the S-layer homology domain was expressed, most of the activity was found in the insoluble fraction (data not shown), demonstrating that the S-layer domain is essential for the solubility of the enzyme.

**DISCUSSION**

In this paper, we describe the isolation, purification, characterization, and molecular cloning of a novel keratan sulfate hydrolase, endo-β-N-acetylglucosaminidase, from B. circulans. Like the keratanase II previously identified in Bacillus sp. KS36, this enzyme cleaves KS chains releasing sulfated N-
acetylated lysamine and therefore is defined as a keratanase II. The deduced amino acid sequence of the enzyme, designated as Be keratanase II, indicated that the gene contains the consensus sequence of the carbohydrate-binding domain in the middle portion and the surface layer homology domain at the C terminus. The full-length protein expressed by a bacterial system has enzyme activity comparable with the native enzyme, and functional analysis of the truncated proteins demonstrated the contribution of the S-layer domain to solubility.

Be keratanase II has at least three advantages compared with the enzyme of Bacillus sp. Ks36. First, B. circulans produces a larger amount of the enzyme, which enables a higher yield and easy purification. Thirty grams of bacterial pellet (wet weight) contains 80 units of activity, compared with 24 units from Bacillus sp. Ks36. Actually, we have successfully purified the native enzyme by using essentially a two-step column chromatography, yielding ~70 mg of protein from 7.5 g of total protein, with ~64-fold purification, whereas keratanase II of Bacillus sp. Ks36 has been purified by a three-step column chromatography by using phenyl-Sepharose, DEAE-cellulofine, and Sephacryl S-300 following ammonium sulfate precipitation, with a purification of ~8,000-fold. Second, Be keratanase II is thermostable with an optimal temperature of 55 °C. Third, the enzyme is unaffected by metal ions, SH inhibitors, and chelating agents, whereas the activity of Bacillus sp. keratanase II is inhibited considerably by Zn2+, Mn2+, and EDTA, and completely by p-chloromercuribenzoic acid.2

With these characteristics as above, keratanase II of B. circulans would be extremely useful for fluorophore-assisted carbohydrate electrophoresis analysis, which is a simple, highly sensitive, and quantitative analysis of KS chains (15). Recent studies revealed that keratanase II preparations of Bacillus sp. Ks36 contained as a contaminant β-galactosidase activity that cleaves the disaccharide Galp1–4GlcNAc6S to Gal and GlcNAc6S (15). So far we have not observed such activity in the purified Be keratanase II, and even if present, depolymerization using this enzyme at a temperature higher than 37 °C may considerably reduce it.

Another possible application of keratanase II is production of sulfated N-acetyllactosamine. The fact that 6-sulfo-N-acetyllactosamine is specific to some classes of dendritic cells suggests an important role for this saccharide structure in T cell stimulation by dendritic cells (5). The N-acetyllactosamine structure has been observed in sialyl Lewis x (Lea), a functional ligand for E-, P-, and L-selectins (20–23), which mediate extravasation of leukocytes. Since sialyl 6-O-sulfo-Lea is a ligand for L-selectin, depolymerized KS oligosaccharides may have a physiological function or may serve as a competitive inhibitor for their binding.

It is intriguing that keratanase II cleaves highly sulfated KS but not the desulfated form. Effects of sulfation levels on enzymatic activity have been demonstrated in other glycosaminoglycan-degrading enzymes such as chondroitinases ABC and ACII. Chondroitin ABC digests chondroitin sulfate rather than chondroitin, whereas chondroitinase ACII digests chondroitin better. However, the selectivity of KS-degrading enzymes appears strict compared with that of chondroitinases. For instance, sulfation of at least one of the GlcNAc residues adjacent to a Gal residue is essential for the action of keratanase of Pseudomonas sp. but not for endo-β-galactosidase (14). By NMR analysis, shark cartilage KS did not appear to contain fucosylated GlcNAc (data not shown). The effect of fucosylation on the substrate specificity remains to be examined.

We have performed molecular cloning of keratanase II for the first time, and we have further expressed an enzyme with comparable activity to the native form, suggesting that this E. coli system may be adapted to expression of glycosidases for bulk production and possible therapeutic use. As the carbohydrate-binding domain located in the middle is also found in the endo-β-galactosidase of F. keratolyticus, this domain may be important for specific recognition of the sulfated KS chains. Functional analysis using various truncated forms will determine the catalytic site and regions responsible for thermostability. At present, the physiological turnover of KS chains has not been well studied, and no KS-degrading enzyme has been identified in mammals. Our cloning data may facilitate identification of a mammalian counterpart of the enzyme, which may lead to elucidation of mechanisms of KS metabolism.

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