Crystal Structure of Unsaturated Glucuronyl Hydrolase Complexed with Substrate: Molecular Insights into Its Catalytic Reaction Mechanism
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Unsaturated glucuronyl hydrolase (UGL), which is a member of the glycoside hydrolase family GH-88, is a bacterial enzyme that degrades mammalian glycosaminoglycans and bacterial biofilms. The enzyme, which acts on unsaturated oligosaccharides with a α-glycoside bond produced by microbial polysaccharide lyases responsible for bacterial invasion of host cells, was believed to release 4-deoxy-L-threo-5-hexosulose-uronate (unsaturated glucuronic acid: ∆GlcA) and saccharide with a new nonreducing terminus by hydrolyzing the glycosidic bond. We detail the crystal structures of wild-type inactive mutant UGL of Bacillus sp. GL1 and its complex with a substrate (unsaturated chondroitin disaccharide), identify active site residues, and postulate a reaction mechanism catalyzed by UGL that triggers the hydration of the vinyl ether group in ∆GlcA, based on the structural analysis of the enzyme-substrate complex and biochemical analysis. The proposed catalytic mechanism of UGL is a novel case among known glycosidases. Under the proposed mechanism, Asp-149 acts as a general acid and base catalyst to protonate ∆GlcA C4 atom and to deprotonate the water molecule. The deprotonated water molecule attacks the ∆GlcA C5 atom to yield unstable hemiketal; this is followed by spontaneous conversion to an aldehyde (4-deoxy-L-threo-5-hexosulose-uronate) and saccharide through hemiacetal formation and cleavage of the glycosidic bond. UGL is the first clarified α6/α6-barrel enzyme using aspartic acid as the general acid/base catalyst.

Glycosaminoglycans such as chondroitin, hyaluronan, and heparin are highly negatively charged polysaccharides with a repeating disaccharide unit consisting of an uronic acid residue (glucuronic or iduronic acid) and an amino sugar residue (glucosamine or galactosamine) (1). Chondroitin consists of D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) along with a sulfate group(s) at position 4 or 6 or at both positions. Hyaluronan, which is also a member of the glycosaminoglycan family, is composed of GlcA and N-acetyl-D-glucosamine (GlcNAc). These major components of the mammalian extracellular matrix are responsible for cell-to-cell association, and are widely present in human tissues such as the eye, brain, liver, skin, and blood (2).

Glycosaminoglycans in the extracellular matrix are enzymatically degraded by hydrolases and lyases (3). Generally, hydrolases, which cleave bonds between glycosyl oxygen and anomeric carbon atoms through the addition of a water molecule, play an important role in the metabolism of glycosaminoglycans in mammals. Microbial pathogens that invade host cells degrade glycosaminoglycans primarily by the action of lyases (4,5). Microbial lyases recognize the uronic acid residue in polysaccharides and produce
unsaturated disaccharides with GlcA residue having a C=C double bond at the nonreducing terminus through the β-elimination reaction. Typical pathogens that degrade glycosaminoglycans include streptococci such as \textit{Streptococcus agalactiae}, \textit{Streptococcus pneumoniae}, and \textit{Streptococcus pyogenes}. Streptococci cause severe infectious diseases, e.g., pneumonia, bacteremia, sinusitis, and meningitis; this bacterium degrades glycosaminoglycans by using polysaccharide lyases, which function as virulence factors (6).

Unsaturated glucuronyl hydrolase (UGL), a member of glycoside hydrolase family GH-88 in the CAZy database (Henrissat, B., Coutinho, P., and Deleury, E., 1999; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), acts on unsaturated oligosaccharides that have an unsaturated GlcA (\(\Delta\)GlcA) with an \(\alpha\)-glycoside bond, i.e., \(\Delta\)GlcA-GalNAc produced by chondroitin lyase, \(\Delta\)GlcA-GlcNAc produced by hyaluronate lyase, \(\Delta\)GlcA-mannose (Man)-glucose (Glc) produced by xanthan lyase, and \(\Delta\)GlcA-Glc-rhamnose (Rha)-Glc produced by gellan lyase (7) (Fig. 1). The enzyme reaction produces \(\Delta\)GlcA, and the leaving saccharide. The resultant product, \(\Delta\)GlcA, is thought to be spontaneously converted to the open chain from because the ringed form of \(\Delta\)GlcA is not obtained due to keto-enole equilibrium (5,7). UGL and its gene were first identified in \textit{Bacillus} sp. GL1 and were considered to be involved in the degradation of mammalian glycosaminoglycans and bacterial biofilms (\textit{Sphingomonas} gellan and \textit{Xanthomonas} xanthan) (7). Proteins showing high homology with UGL of \textit{Bacillus} sp. GL1 were subsequently found to be encoded in the genomes of pathogenic bacteria, i.e., bacteroides, enterococci, mycoplasma, streptococci, and vibrio (supplementary figure, Fig. S1). The enzyme thus appears to be responsible for the complete degradation of glycosaminoglycans. In fact, streptococcal hypothetical proteins homologous to UGL were recently found to exhibit enzyme activity (Hashimoto \textit{et al.}, in preparation).

Structural analysis of lyases and UGL is indispensable for clarifying molecular mechanisms underlying the recognition and degradation of unsaturated saccharides and sequential reaction mechanisms involved in polysaccharide depolymerization by bacteria. The crystal structures of polysaccharide lyases acting on chondroitin AC (8,9), chondroitin B (10), and chondroitin ABC (11), hyaluronate (12-15), and xanthan (16,17) have been determined, and the structural and functional relationships of these lyases have been studied. We recently determined the crystal structure of UGL (PDB, Accession No.: 1VD5) and showed that it includes a \(\alpha_6/\alpha_6\)-barrel topology, which is found in the \(\alpha/\alpha\)-toroidal fold in the \(\alpha/\alpha\)-toroidal fold in the six-hairpin glycosidase superfamily (18). The \(\alpha/\alpha\)-toroidal fold is also found in polysaccharide lyases. One side of the \(\alpha/\alpha\)-barrel structure consists of long loops and contributes to the formation of a deep pocket. In glycosidases, two carboxyl and carboxylate groups are reported to generally function as catalytic residues (19,20), and, based on structural and mutagenesis studies, two candidate residues (Asp-88 and Asp-149) were proposed to be located in the pocket (18). The catalytic reaction mechanism of the enzyme remains to be elucidated, however.

The previous crystal structure (1VD5) of the enzyme showed that one glycine and two dithiothreitol (DTT) molecules derived from a crystallization solution are bound in the deep pocket of the enzyme. In this study, we detail the determination of crystal structures of a mutant UGL and its complex with a substrate (\(\Delta\)GlcA-GalNAc), identification of active site residues, and the proposed catalytic reaction mechanism. More recently, we also studied the substrate specificity and recognition mechanism of UGL using the structures of other enzyme-substrate complexes (\(\Delta\)GlcA-Glc-Rha-Glc and \(\Delta\)GlcA-GlcNAc; PDB accession No. 2FVO and 2FV1) (21). These structures enabled us to clarify the novel catalytic reaction mechanism involved in the degradation of unsaturated saccharides by GH-88 family enzymes.
Experimental Procedures

Crystallization and X-ray diffraction - Wild-type and a UGL mutant having Asp-88 substituted with Asn (D88N) were overexpressed in *Escherichia coli* and purified as described previously (18). The purified enzymes were concentrated by vivaspin 0.5 ml concentrators with 10,000 MWCO membranes (Vivascience, Hannover, Germany) to a final concentration of 10 mg/ml. The concentrated enzymes, in 20 mM potassium phosphate (pH 7.0), were then used in the crystallization step. Crystals were obtained by using hanging drop vapor diffusion and microseeding. Hanging drops were prepared by mixing 3 µl of the enzyme solution with an equal volume of reservoir solution containing 40% (w/v) polyethylene glycol 10000 and 0.15 M Tris-HCl (pH 8.6), and equilibrated with 0.5 ml of the reservoir solution at 20°C. After two weeks, minute crystals formed in drops. These initial crystals were crushed by a stainless steel needle and transferred to hanging drops equilibrated under conditions similar to those used for initial crystals, except for 25% (w/v) polyethylene glycol 10000. Rod-shaped crystals grew to a maximum 0.1 × 0.1 × 0.5 mm during the three months after seeding. One D88N crystal was soaked in the substrate solution containing 400 mM ΔGlcA-GalNAc (chondroitin disaccharide di-0S sodium salt; Sigma, St. Louis, MO, USA), 25% (w/v) polyethylene glycol 10000, and 0.15 M Tris-HCl (pH 7.5) for 10 min at 20°C. The pH optimum of the enzyme is about pH 6. pK_a2 is about pH 7.6. We therefore moved crystals used here into the solution containing Tris pH 7.5. We therefore moved crystals used here into the solution containing Tris pH 7.5. Crystals were placed in a cold nitrogen gas stream at −173°C. X-ray diffraction images of crystals were collected using a JUPITER 210 CCD detector (Rigaku, Tokyo, Japan) with synchrotron radiation at a wavelength of 0.80 Å (D88N/ΔGlcA-GalNAc complex and wild-type) at the BL-38B1 station of SPring-8 (Hyogo, Japan). Images were processed using DENZO and SCALEPACK software (22) to a resolution of 1.60, 1.52, or 1.90 Å (Table I).

Structure determination and refinement - Structures were determined using molecular replacement and refined using CNS program ver. 1.1 (23) with the previously reported UGL structure (1VD5) as the reference model. Several rounds of positional and B-factor refinement were conducted, followed by manual model building using the TURBO-FRODO program (AFMB-CNRS, Marseille, France) to improve the model by increasing data to a resolution of 1.60, 1.52, or 1.90 Å for wild-type, D88N, and D88N/ΔGlcA-GalNAc with the CNS program (Table I). The stereoequality of the model was assessed using the PROCHECK (24) and WHATCHECK (25) programs. Structural alignment was conducted by superimposition using a fitting program in TURBO-FRODO. Ribbon plots were prepared using the MOLSCRIPT (26), RASTER3D (27), and GRASP (28) programs. Carbohydrate conformations (puckering parameters) as defined by Cremer and Pople (29) were analyzed using the PLATON program (30).

Site-directed mutagenesis - Arg-221, His-193, or Gln-211 in UGL was replaced with an alanine residue by using a QuickChange site-directed mutagenesis kit (Stratagene Co., CA, USA); mutations were confirmed by DNA sequencing. The plasmid pET3a-UGL, which is the expression vector for wild-type UGL (7), was used as a template. The following primers were used: R221A (Arg-221 → Ala-221), 5′-AGCACGTGGACGGCCAGGCTTG-3′ and 5′-CCAAGCCTGGCCGGCCGTCCAGGTGCTTCTGAATCCACC-3′; H193A (His-193 → Ala-193), 5′-CGACGATTGAGCTATGGGACGTCTTCTTA and 5′-GGTCCAGAAGTAGACGGCAAGTCCAGCTCATAAGCTC-3′; and Q211A (Gln-211 → Ala-211), 5′-GGCGGCACGCAGCGCCGCCGAACACC and 5′-GTCGGTGTTGCCCCGGGTGCTGCCG-3′ (mutations are indicated by bold letters). Cells of *E. coli* BL21(DE3) were transformed with mutant plasmids, i.e., pET3a-UGL(R221A), pET3a-UGL(H193A), and pET3a-UGL(Q211A).
and pET3a-UGL(Q211A). Mutant enzymes were expressed and purified using a procedure similar to that used for wild-type UGL (7). Enzyme purity was assessed by SDS-PAGE followed by Coomassie Brilliant Blue staining.

**Enzyme assay** - Enzyme-substrate reactions for the wild-type enzyme and mutants were conducted at 30°C as follows: the reaction mixture consisted of 50 mM sodium phosphate (pH 6.5), the substrate, and enzyme in a 500 µl reaction volume. Since a large amount of glycosaminoglycan disaccharides was unavailable, gellan tetrasaccharide (ΔGlcA-Glc-Rha-Glc) produced from gellan by gellan lyase was used as the UGL substrate (7). Enzyme activity was measured by monitoring the decrease in absorbance at 235 nm, corresponding to the loss of the C=C double bond of the substrate (Fig. 1d) (7). \( k_{cat} \) and \( K_m \) parameters were determined by nonlinear fitting to the Michaelis–Menten equation. Enzyme concentration was determined by UV spectrophotometry by using theoretical molar extinction coefficient \( \varepsilon_{280} = 99,570 \) (M⁻¹cm⁻¹). The dependence of \( k_{cat}/K_m \) on pL (pH or pD) was measured in solutions consisting of 50 mM acetate pL 3.5 – 5.5 or sodium phosphate pL 5.2 - 8.2. pD values were estimated by adding 0.41 to the pH meter reading (31). The dependence of \( k_{cat}/K_m \) on pL was fitted to the following equation: \( (k_{cat}/K_m)_{obs} = (k_{cat}/K_m)_{0}/(1 + 10^{pK_{a1} - pL} + 10^{pL - pK_{a2}}) \). The solvent kinetic isotope effect (SKIE) on \( k_{cat} \) and \( K_m \) was measured near the optimum pL (pH 6.0 or pD 6.6).

**Gas chromatography/mass spectrometry (GC/MS) analysis** - Enzyme reaction mixtures [2 µmol sodium phosphate (pH 6.5), 2.5 mg ΔGlcA-GalNAc, and 30 µg UGL in a 100 µl reaction volume with 88, 50%, 0% 18O water (18O, 95%, Cambridge Isotope Laboratories, MA, USA)] were incubated at 30°C overnight. Enzyme molecules in reaction mixtures were removed by vivaspin 0.5 ml concentrators with 10,000 MWCO membranes. Reduction, methylation, and GC/MS analysis were conducted based on the method of Chai et al. (32) as follows: After filtrated products (10 µl) were lyophilized and incubated with 0.5 ml of 1% NaBH₄ at room temperature overnight, excess NaBH₄ was decomposed by 50% acetic acid. Reduced products were purified five times with 100% methanol. Methylation was conducted by the NaOH-CH₃I method with reduced products (33). Methylated products were extracted with chloroform, dried with Na₂SO₄, and redissolved in chloroform for GC/MS analysis. GC/MS analysis was conducted on a Hewlett Packard HP5890A gas chromatograph (Agilent Technologies, CA, USA) with a JMS-DX303 mass spectrometer (Jeol, Tokyo, Japan) and a Supelco SPB-5 capillary column (31 m x 0.25 mm i.d. x 0.25 µm film thickness; Sigma-Aldrich Corporate, MO, USA) with He as the carrier gas. The initial column temperature of 60°C was held for 1 min and increased to 280°C at 8°C/min. Mass spectra (m/z 35 – 500) were acquired at 70 eV, 300 µA, and a source temperature of 250°C.

**Results and Discussion**

**Structure determination** - UGL of Bacillus sp. GL1 is a monomeric enzyme with a molecular mass of approximately 43 kDa (377 amino acid residues) (7). The hexagonal crystal structure of wild-type UGL has been reported previously. This structure contains one glycine and two DTT molecules derived from the crystallization solution in the deep pocket (1VD5), making it difficult to determine the structure of the enzyme-substrate complex (18). Asp-88 is located in the deep pocket, and is completely conserved in UGL and its homologues (supplementary figure, Fig. S1). The molecular activity (\( k_{cat} = 0.00057 \) s⁻¹) of mutant D88N is about 13000-fold lower than that (\( k_{cat} = 7.3 \) s⁻¹) of the wild-type enzyme, although the Michaelis constants (Km) of both are comparable (18). Due to the lack of enzyme activity, a mutant having an affinity for the substrate has a greater advantage in forming the enzyme-substrate complex than the wild-type enzyme. We attempted to crystallize this inactive mutant under conditions differing from those in the
previous study (18). Orthorhombic crystals (wild-type and D88N) were obtained by hanging drop vapor diffusion and microseeding by using polyethylene glycol 10000 as a precipitant.

\( \Delta \text{GlcA-GalNAc} \) produced from chondroitin by chondroitin lyase is a disaccharide and a good substrate for UGL (Fig. 1a) (18). The structure of the D88N/substrate complex was first determined using \( \Delta \text{GlcA-GalNAc} \) (D88N/\( \Delta \text{GlcA-GalNAc} \)). Other complex structures (D88N/GlcA-Glc-Rha-Glc and D88N/GlcA-GalNAc) were detailed for the substrate recognition mechanism of UGL (21). The results of data collection for structures using synchrotron radiation are summarized in Table I.

Refined models consist of 754 (377 \( \times \) 2 monomers) amino acid residues and 2 \( \Delta \text{GlcA-GalNAc} \) molecules (Fig. 2a). Two identical monomers are in an asymmetric unit, designated A- and B-molecules for convenience. The two \( \Delta \text{GlcA-GalNAc} \) models are well fitted, but the average \( B \)-factor of the B-molecule is higher than that of the A-molecule (Table I). The \( \Delta \text{GlcA-GalNAc} \) (A) is better characterized than the B-molecule. \( \Delta \text{GlcA} \) (A) is particularly well settled (Fig. 2b).

Structures of orthorhombic crystals (wild-type, D88N, and D88N/\( \Delta \text{GlcA-GalNAc} \)) coincide, except for an active site, described later. The structures are very similar to the hexagonal crystal structure of the wild-type enzyme, showing no significant difference from the overall structure. The enzyme molecule is approximately 45 \( \times \) 45 \( \times \) 40 Å and has an \( \alpha_6/\alpha_6 \)-barrel structure that consists of 12 long \( \alpha \)-helices (H1–H12, 51% of all residues), 3 antiparallel \( \beta \)-sheets consisting of 2–3 strands (SA–SC, 6.6%), 2 short 3\( \alpha \)-helices adjoining the long \( \alpha \)-helices, and some loops (Fig. 2a), as described previously (18). The barrel structure forms a deep pocket consisting of long loops and \( \beta \)-sheets, where the substrate is bound in D88N/\( \Delta \text{GlcA-GalNAc} \). The deep pocket, approximately \( \sim \)20 Å in diameter at the lip and \( \sim \)15 Å deep, is strongly suggested to be an active site.

\( \Delta \text{GlcA-GalNAc} \) bound at the active site

- The structure of the enzyme-substrate complex contains one substrate, the \( \Delta \text{GlcA-GalNAc} \) molecule, per monomer. Two planes of sugar rings are almost parallel with the axis of symmetry of the \( \alpha_6/\alpha_6 \)-barrel structure (Figs. 2a and 3). The substrate is bound at the bottom of the deep pocket, indicating that it occupies two subsites, \(-1 \) and \(+1 \), at the active site. The nomenclature of subsites complies with that described by Davies et al. (34). \( \Delta \text{GlcA-GalNAc} \) is bound at the surface surrounded by aromatic amino acid residues (Phe, Trp, and Tyr) (Fig. 3a). The puckering parameters (29) of bound \( \Delta \text{GlcA-GalNAc} \) were \( Q = 0.52 \) Å, \( \Theta = 50^\circ \), and \( \Phi = 85^\circ \) for \( \Delta \text{GlcA} \), and \( Q = 0.59 \) Å, \( \Theta = 4^\circ \), and \( \Phi = 354^\circ \) for GalNAc. \( \Delta \text{GlcA} \) has a half-chair configuration (\( ^2H_1 \)) and GalNAc a stable chair configuration (\( ^1C_4 \)). The C3, C4, and C5 atoms of \( \Delta \text{GlcA} \) are on the same plane as the C4–C5 double bond. \( \Delta \text{GlcA} \) therefore has an unusual configuration. The dihedral angles of the \( \alpha-1,3 \)-linkage between \( \Delta \text{GlcA} \) and GalNAc are \( \varphi (O5_{\Delta \text{GlcA}} - C1_{\Delta \text{GlcA}} - O3_{\text{GalNAc}} - C3_{\text{GalNAc}}) = -74^\circ \) and \( \psi (C1_{\Delta \text{GlcA}} - O3_{\text{GalNAc}} - C3_{\text{GalNAc}} - C3_{\text{GalNAc}}) = -146^\circ \). These torsion angles are preferable and stable in the lowest energy region of the chondroitin saccharide isoelectrostatic map (35).

The number of interactions between D88N and GalNAc is fewer than that between D88N and \( \Delta \text{GlcA} \) (Fig. 3b and Table II). UGL also acts on other unsaturated oligosaccharides such as \( \Delta \text{GlcA-GlcNac} \), \( \Delta \text{GlcA-Man-Glc} \), and \( \Delta \text{GlcA-Glc-Rha-Glc} \) (Fig. 1). Although UGL activity toward these substrates differs to some extent (18), all of these substrates contain \( \Delta \text{GlcA} \) at the nonreducing terminus. It appears that subsite \(+1 \) can accommodate various saccharides. In other words, UGL strictly recognizes \( \Delta \text{GlcA} \) at subsite \(-1 \) rather than the saccharide at subsite \(+1 \). This is supported by the fact that the average \( B \)-factor of \( \Delta \text{GlcA} \) is significantly lower than that of GalNAc (Table I).

\( \Delta \text{GlcA} \) interacts with Trp-42, Asn-88, Phe-91, Trp-134, Asp-149, Gln-211,
Trp-219, Arg-221, Trp-225, and Tyr-338 residues (Fig. 3b and Table II). All of these residues are completely conserved in UGL and its homologues (supplementary figure, Fig. S1). In particular, the aromatic side chain of Trp-42 shows a stacking interaction with the sugar ring of \(\Delta\)GlcA and plays an essential role in binding \(\Delta\)GlcA. The side chain of Trp-225 forms a hydrogen bond to the carboxyl group of \(\Delta\)GlcA. Other aromatic side chains partially interact with the \(\Delta\)GlcA pyranose ring hydrophobically. The positively charged side chain of Arg-221 faces the carboxyl groups of \(\Delta\)GlcA. The two O atoms (O6A and O6B) of the carboxyl group form hydrogen bonds directly with Arg-221, Gln-211, and Trp-225. Through direct hydrogen bonds, O2 and O3 atoms associate with Asn-88, Asp-149, and Trp-134 at the bottom surface. The O5 atom interacts with Asp-149, His-193, and Gln-211 through a water molecule (Wat-1). For some polysaccharide lyases such as chondroitin lyase (9), hyaluronate lyase (14), and xanthan lyase (17), the asparagine residue is close to the carboxyl group and is important for catalysis because it acts to neutralize the negative charge of the carboxyl group. Thus, to determine the effect of Arg-221 on the substrate, we prepared and assayed the mutant enzyme, R221A (Table III). The kinetic parameters of R221A are comparable to those of the wild-type enzyme, suggesting that the interaction between Arg-221 and the carboxyl group of \(\Delta\)GlcA is not essential to bind the substrate or to participate in catalysis. Other than Arg-221, there are two residues (Gln-211 and Trp-225) forming hydrogen bonds to this group. \(\Delta\)GlcA is bound at the positive end of the inner \(\alpha\) helix of the \(\alpha\)6/\(\alpha\)6-barrel, suggesting that two residues (Gln-211 and Trp-225) and a helix dipole would be more effective for neutralizing the substrate than the side chain of Arg-221. UGL cannot act on the substrate with GlcA at the nonreducing terminus (7); this suggests that the presence of the C4 hydroxyl group of GlcA may inhibit enzyme action due to unsuitable accommodation at subsite –1, but the superimposition of GlcA on \(\Delta\)GlcA shows that no three-dimensional obstacle particularly surrounds the C4 hydroxyl group. The possible reason for the inability of UGL to act on usual glucuronyl saccharides is discussed later.

GalNAc interacts with Trp-42, His-86, Trp-134, His-210, Tyr-338, and His-339 residues (Fig. 3b and Table II). The N-acetyl group of GalNAc (N2, C7, C8, and O7 atoms) has few interactions with the enzyme and is thus disordered (Fig. 2b and Table II). Another substrate for UGL has Man or Glc residue at subsite +1, which contains no such group (Fig. 1). Two residues (His-210 and His-339) in UGL are replaced with arginine and serine in other homologues (supplementary figure, Fig. S1). Based on the broad specificity of subsite +1 and conserved residues, the hydrophobic moiety, e.g., the pyranose ring, is important for accommodation at subsite +1 through hydrophobic interactions with aromatic side chains of Tyr-338 and Trp-134, although some hydrogen bonds are present in binding GalNAc.

Comparison to the substrate-free structure

- The overall structures of the wild-type enzyme (old hexagonal crystal form and new orthorhombic one), D88N, and D88N/\(\Delta\)GlcA-GalNAc are essentially identical as stated above. In the deep pocket, they share structures almost similar except for the side chains of Asp-149 and Trp-134, which move based on ligand binding (Fig. 4). Although the side chain of Asp-149 was modeled with two alternate conformations in the wild-type/apo form (Fig. 4a, wild-type model), it is fixed with the ligand in the holo form by rotating about 85° (Fig. 4, D88N/\(\Delta\)GlcA-GalNAc and Fig. 4b hexagonal wild-type models). The side chain of Trp-134 is also fixed based on ligand binding by rotating about 110°. Although apo forms (orthorhombic wild-type and D88N) have more water molecules at the ligand than the complex form (D88N/\(\Delta\)GlcA-GalNAc and hexagonal wild-type), the Wat-1 molecule is located at the same position in all structures (Figs. 3b and 4). The hexagonal wild-type structure has one additional glycine residue, two DTT molecules derived from the crystallization solution, and two water...
molecules at the active site (Fig. 4b). The location of the carboxyl group of the glycine molecule in the wild-type enzyme corresponds to that of ∆GlcA in substrate-bound D88N, and both carboxyl groups interact with the enzyme identically. Two hydroxyl groups (O2 and O3) of ∆GlcA that form hydrogen bonds with Asn-88 are replaced with two additional water molecules bound to Asp-88 through hydrogen bonds. One DTT molecule is located at subsite +1 corresponding to the position of GalNAc, and has hydrophobic interactions with Tyr-338. The second DTT molecule interacts mainly with the aromatic side chain of Trp-134.

**Catalytic mechanism** - UGL acts strictly on unsaturated oligosaccharides with ∆GlcA at the nonreducing terminus, and is inert on saccharides containing GlcA (Fig. 1) (7). Products obtained through the enzyme reaction are 4-deoxy-L-threo-5-hexosulose-uronate and the leaving saccharide with a new nonreducing terminus (Fig. 1d) (5,7). To the best of our knowledge, general β-glycosidase cannot act on unsaturated saccharides with ∆GlcA at the nonreducing terminus. The reaction mechanism of the hydrolysis of these unsaturated saccharides thus remains unclear.

The structure of UGL in a complex with the substrate (D88N/∆GlcA-GalNAc) determined in the present study may provide valuable clues to the nature of the catalytic reaction mechanism. Regarding the binding of ∆GlcA to the active site, no atom is located close to glycosidic oxygen (O3 atom of GalNAc) within the hydrogen bond distance (< 3.5 Å). The characteristic environment of the active site is as follows (Fig. 3b): a water molecule (Wat-1) is present at the “α-face position” of the ∆GlcA pyranose ring and forms four hydrogen bonds with Asp-149 (3.1 Å), His-193 (2.7 Å), Gln-211 (2.6 Å), and O5 atom (3.2 Å) of ∆GlcA, but not with glycosidic oxygen (4.2 Å); Wat-1 is also located close to the C5 carbon atom (3.4 Å) but not to the C1 carbon atom (3.9 Å); Asp-149 is close to the C4 atom (2.9 Å) but not to glycosidic oxygen (5.8 Å) and C1 carbon (4.8 Å) atoms; Asn-88 located at the mutation site (wild-type, Asp-88) is also far from glycosidic oxygen (4.9 Å) and C1 carbon (4.3 Å) atoms; no water molecule exists at the “β-face position” of the pyranose ring; ∆GlcA has an alkene C4=C5 double bond, enriched in electrons and termed an “electron sink.” These characteristics are unusual in glycoside hydrolases (19,20). In particular, no hydrogen bonds are formed with glycosidic oxygen. We therefore postulate a catalytic reaction mechanism that involves water addition reaction (hydration) of the vinyl ether group (C4=C5–O5) and subsequent hydrolysis of the glycosidic bond in ∆GlcA. This postulation is based on the structure of the enzyme-substrate complex, the characteristic of the substrate [i.e., susceptibility of the vinyl ether group with an “electron sink” in ∆GlcA to the water addition reaction (36,37)], enzyme properties determined through kinetics on site-directed mutants as described later. A possible catalytic reaction mechanism of UGL is shown in Fig. 5. Asp-149 acts as a general acid catalyst and donates a proton to the double bond (C4 atom). Asp-149 acts as a general base catalyst and deprotonates the water molecule (Wat-1); the deprotonated water molecule then attacks the C5 atom of ∆GlcA. The product hemiketal is unstable and readily converts to α-keto acid (hemiacetal). Due to aldehyde-hemiacetal equilibrium, the resultant hemiacetal finally converts to the aldehyde (4-deoxy-L-threo-5-hexosulose-uronate) and the leaving saccharide through cleavage of the glycosidic bond. These consecutive reactions appear to be similar to the oxymercuration of unsaturated hyaluronate disaccharide ∆GlcA-GlcnAc (38). In other words, the water addition reaction (hydration) of the vinyl ether group in ∆GlcA and spontaneous conversion of hemiketal to aldehyde and the leaving saccharide through cleavage of the glycosidic bond. These consecutive reactions appear to be similar to the oxymercuration of unsaturated hyaluronate disaccharide ∆GlcA-GlcnAc (38). In other words, the water addition reaction (hydration) of the vinyl ether group in ∆GlcA and spontaneous conversion of hemiketal to aldehyde and the leaving saccharide via α-keto acid (hemiacetal) are also demonstrated in the reaction mixture of ∆GlcA-GlcnAc and mercuric salts.

Many glycosidases hydrate C1 anomeric configuration-free unsaturated substrates (D-glycals, C2=C1-O5 or D-glyco-enitols, O5-C1=C). Their catalytic
mechanism and anomeric configuration of products are regulated by their own specificities in hydrolysis, such as “retaining” or “inverting mechanisms” (39-41). UDP-GlcNAc 2-epimerase catalyzes the epimerization of UDP-GlcNAc and UDP-N-acetylmannosamine (UDP-ManNAc) in bacteria, or the conversion of ManNAc and UDP in mammals. Their reactions involve elimination to generate a vinyl ether group (2-acetamidoglucal: C2=C1-O5) and the readdition of UDP in bacteria or a water molecule in mammals (42,43). The proposed mechanism of UGL is similar. In particular, the carboxylate group (Asp-149: UGL) acting as the general acid/base catalyst to protonate the vinyl group and deprotonate the catalytic water molecule is equivalent to those of retaining glycosidases. The reaction mechanism consisting of the hydration of the vinyl ether group (C4=C5-O5) triggering hydrolysis of the glycosidic bond and the arrangement of the active site residue, however, are novel and intriguing (Figs. 3b and 5).

The location of Asp-149 is suitable for the protonation of the C4 atom of ∆GlcA (Fig. 3b), and Asp-149 is completely conserved in UGL and its homologues (supplementary figure, Fig. S1). A mutant enzyme D149N shows significantly reduced molecular activity (0.12%) when compared to the wild-type enzyme, as described previously (18). Asp-88 is thought to both stabilize the carbenium ion at C5 of ∆GlcA during catalysis and mainly to tie protons of hydroxyl groups of O2 and, most importantly, O3 in hydrogen bonds (Figs. 3b and 5). Otherwise, the O3 hydroxyl group would be able to form a hydrogen bond to the side chain of Asp-149, preventing its protonation and also directing it away from C4 and toward O3 (Fig. 3b).

We measured the SKIE to confirm the participation of the general acid and water molecule in the reaction. The SKIE on the second-order rate constant (kcat/Km) of UGL depends on the pL (pH or pD). The optimal pL (pH = 6.0 or pD = 6.6) and the two pKa values (pKa1 = 4.4 or 5.2, pKa2 = 7.6 or 8.2) increased in D2O. The shift of 0.6 units in pKa2, thought to be pKa of the side chain of Asp-149, is typically seen for acids (44). There is a significant SKIE on both kcat and kcat/Km near the optimal pL. Catalysis in H2O (pH 6.0) showed kcat = 6.8 ± 0.7 (s⁻¹), Km = 62 ± 13 (µM), and kcat/Km = 0.11 (µM⁻¹s⁻¹); that in D2O (pD 6.6) gave kcat = 3.3 ± 0.2 (s⁻¹), Km = 66 ± 11 (µM), and kcat/Km = 0.05 (µM⁻¹s⁻¹). The SKIE is thus 2.1 for kcat and 2.2 for kcat/Km. General acid
catalysis is associated with the SKIE for $k_{cat}$ of 1.5-2.5 (45). These observed SKIEs are typical for general acid catalysis and indicate the mechanism involving proton transfer in the rate-determination step (46).

The structural basis for hydration of the intrinsic substrate with the vinyl ether group through enzymatic catalysis by the asparagine residue has been reported with isochorismatase (PhzD) from *Pseudomonas aeruginosa* (47). PhzD is involved in phenazine biosynthesis and catalyzes the hydrolysis of the vinyl ether group of 2-amino-2-deoxyisorhamnate. Although UGL and PhzD show no similarity in amino acid sequence or overall structure, their amino acid residues interacting with the substrate are comparable as follows: the catalytically important residue (Asp-149) in UGL is equivalent to Asp-38 in PhzD, and the other important residues (Trp-42 and Tyr-338) in UGL correspond to those of Trp-94 and Tyr-151 in PhzD. Asp-38 in PhzD is considered to be a general acid catalyst and is thought to hydrate the vinyl ether group (47). The magnitude of $k_{cat}$ (~10 s$^{-1}$) of UGL is close to that of PhzD. This evidence suggests that both UGL and PhzD have a similar enzyme reaction mechanism (hydration), and that the protonation of the vinyl ether group by aspartate residues is the rate-limiting step of the reaction.

The detailed structural analysis of the reaction product of UGL has not been clarified, although the product has been confirmed to be a keto acid derived from $\Delta$GlcA (5,7). We conducted GC/MS analysis on the reaction product. Supplementary figure Fig. S2 shows the electron ionization mass spectrum of reduced and methylated $\Delta$GlcA after the UGL reaction in 0, 50, or 88% $^{18}$O water. The fragmentation pattern of this product (m/z 101, 129, 133, 141, 161, 173, and 191) was equivalent to that of reduced and methylated $\Delta$GlcA released thorough the reaction of unsaturated heparin disaccharide and mercuric salts (32) (supplementary figure, Fig. S2). Only three ion peaks (m/z 105, 163 and 193) were observed, however, with the $^{18}$O water hydrolysis sample, and these peak heights increased with an increasing $^{18}$O water ratio (supplementary figure, Fig. S2b, c). Further fragmentation ion peaks (m/z 73 and 131) produced by the loss of methanol from m/z 105 and 163 were difficult to interpret, possibly due to their low content, the several methyl groups, and stable isotope ion peaks. The 2 mass differences in the three ion peaks between $H_2^{16}$O and $H_2^{18}$O reactions correspond to those between $^{16}$O and $^{18}$O. No 2 mass different ion peak was observed except m/z 105, 163, and 193, and no m/z 135 (133 + 2) ion was observed (supplementary figure, Fig. S2). These fragmentation ion peaks may suggest that $^{18}$O existed on the C5 atom of $\Delta$GlcA but not on the C1 atom after hydrolysis with $^{18}$O water.

In conclusion, the structure of UGL in a complex with unsaturated chondroitin disaccharide provides the basis for substrate binding and insights into the novel catalytic reaction mechanism for glycoside hydrolase. Although the chemical mechanism of acid-catalyzed hydration of the vinyl ether group and the similar mechanism in glycosidases are well characterized, the reaction mechanism involving the hydration of the vinyl ether group triggering the hydrolysis of the glycosidic bond is novel and intriguing. Almost all glycoside-related enzymes (hydrolases and phosphorylase) with a $\alpha_6/\alpha_6$-barrel (six-hairpin glycosidase superfamily of the SCOP database; http://scop.mrc-lmb.cam.ac.uk/scop), such as glucoamylases, endoglucanase CelD, endoglucanase CelA, endo/exocellulase E4, and maltose phosphorylase, are “inverting glycosidases.” The CAZy database divides these enzymes into two clans, i.e., clan GH-L (glucoamylase and maltose phosphorylase) and GH-M (cellulase), based on the active site architecture. UGL is therefore the first $\alpha_6/\alpha_6$-barrel enzyme with hydration of the vinyl ether group and hence may be classified into a new clan.
References

Structure of UGL complexed with substrate

FOOTNOTES

# These authors contributed equally to this work.

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Protein Data Bank accession number - Coordinates of wild-type (apo form), D88N, and D88N/∆GlcA-GalNAc structures are deposited in the RCSB Protein Data Bank under accession numbers 2D5J, 2AHF, and 2AHG.

Abbreviations used are:
UGL, unsaturated glucuronyl hydrolase; ∆GlcA, unsaturated D-glucuronic acid; GlcA, D-glucuronic acid; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; Man, D-mannose; Glc, D-glucose; Rha, L-rhamnose; DTT, dithiothreitol; D88N, UGL mutant having Asp-88 substituted with Asn; D88N/∆GlcA-GalNAc, D88N complexed with chondroitin disaccharide; R221A, UGL mutant having Arg-221 substituted with Ala; H193A, UGL mutant having His-193 substituted with Ala; Q211A, UGL mutant having Glu-211 substituted with Ala; SKIE, solvent kinetic isotope effect; GC/MS, gas chromatography/mass spectrometry; ManNAc, N-acetyl-D-mannosamine; D149N, UGL mutant having Asp-149 substituted with Asn; PhzD, isochorismatase.

Figure Legends

Fig. 1. Structures of unsaturated oligosaccharides. (a) Unsaturated chondroitin disaccharide (∆GlcA-GalNAc), (b) unsaturated hyaluronate disaccharide (∆GlcA-GlcNAc), (c) unsaturated xanthan trisaccharide (∆GlcA-Man-Glc), and (d) unsaturated gellan tetrasaccharide (∆GlcA-Glc-Rha-Glc). Arrows indicate the cleavage site of UGL. The dashed-line arrow indicates UGL hydrolysis. The product is 4-deoxy-L-threo-5-hexosulose-uronate.

Fig. 2. (a) Overall structure of UGL bound with substrates. Two α6/α6-barrels are shown in red (A-molecule) and gray (B-molecule). Two yellow CPK models indicate ∆GlcA-GalNAc. Other colors denote secondary structure elements (cyan and magenta, β-strands; yellow and blue, loops). (b) Electron density of the ∆GlcA-GalNAc substrate in the omit (Fo-Fc) map calculated without the substrate and contoured at the 2.5 σ (magenta) and 3.0 σ (cyan) levels. Characters indicate atom names of the substrate.

Fig. 3. The structure of UGL bound with the substrate at the active site. (a) The structure is represented as a white molecular surface model (magenta, Trp-42, Trp-134, and Tyr-338; red, Asn-88 and Asp-149; blue, Arg-221; green, Gln-211). The bound substrate (∆GlcA-GalNAc) is represented by yellow bond models. (b) Residues interacting with the substrate are represented by bond models. Side chains are indicated in red (Asp and Asn), blue (Arg), purple (Trp, Tyr, Phe), cyan (His), and green (Gln). The substrate is denoted by a yellow bond model (oxygen atom, red; carbon atom, black; nitrogen atom, blue). Two water molecules (Wat-1 and Wat-2)
are represented as light blue balls. Hydrogen bonds are shown as dashed lines.

**Fig. 4.** (a) Structural comparison of wild-type, D88N, and D88N/ΔGlcA-GalNAc complex structures at the active site. Amino acid residues of wild-type are shown in blue, D88N in green, and D88N/ΔGlcA-GalNAc in magenta. The substrate (ΔGlcA-GalNAc) structure is denoted by a yellow bond model (oxygen atom, red; carbon atom, black; nitrogen atom, blue), similar to Fig. 3. Water molecules of wild-type (cyan), D88N (deep green), and D88N/ΔGlcA-GalNAc (red) are represented as ball models. Hydrogen bonds in wild-type and D88N are shown as dashed lines. (b) Structural comparison of the D88N/ΔGlcA-GalNAc complex and wild-type/glycin-DTT complex structures at the active site. The wild-type complex structure is indicated in blue. Glycine and DTT molecules of the wild-type complex structure are denoted by green bond models (oxygen atom, red; carbon atom, black; nitrogen atom, blue; sulfur atom, yellow). Water molecules of the wild-type complex are shown as purple ball models. Hydrogen bonds in the wild-type complex structure are shown as dashed lines.

**Fig. 5.** Proposed catalytic reaction mechanism of UGL. The catalytic reaction proceeds through the water addition reaction of the vinyl ether group, as described in the text. Important residues surrounding the substrate are shown.
Structure of UGL complexed with substrate

Table I Data collection and refinement statistics

<table>
<thead>
<tr>
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<th>Wild-type/D88N</th>
<th>D88N/ΔGlcA-GalNAc</th>
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<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
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<tr>
<td><strong>Unit cell parameters (Å)</strong></td>
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<td>a = 87.9, b = 95.4, c = 95.3</td>
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<td><strong>Data collection</strong></td>
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<td></td>
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<td><strong>Resolution limit (last shell)</strong> (Å)</td>
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<td>50.0–1.52 (1.57–1.52)</td>
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<td>631135 (41083)</td>
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<tr>
<td><strong>Unique reflections</strong></td>
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<td>119866 (11412)</td>
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<td><strong>Redundancy</strong></td>
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<td>5.3 (3.6)</td>
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<td><strong>Completeness ([I]−σ[I]) (%)</strong></td>
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<td>97.2 (93.6)</td>
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<tr>
<td><strong>I/σ(I)</strong></td>
<td>16.4 (2.3)</td>
<td>16.5 (1.2)</td>
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<td><strong>Rmerge (%)</strong></td>
<td>7.9 (27.6)</td>
<td>6.5 (39.2)</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td><strong>Final model</strong></td>
<td>754 (377 × 2) residues, 835 water molecules</td>
<td>754 (377 × 2) residues, 866 water molecules</td>
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<td>37.5, 57.3</td>
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<tr>
<td><strong>DGlcA-GalNAc (B) (DGlcA, GalNAc)</strong></td>
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<td>37.5, 57.3</td>
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<td><strong>R-factor (%)</strong></td>
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<td><strong>Rfree (%)</strong></td>
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<td><strong>Most-favored regions</strong></td>
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*Data in highest resolution shells is given in parentheses.

b $R_{\text{merge}} = \sum \bar{I}_i - \langle I \rangle / \sum \bar{I}_i \times 100$, where $I_i$ is the intensity of individual reflection and $\langle I \rangle$ is the mean intensity of all reflections.

c $R$-factor = $\Sigma|F_o - F_c|/\Sigma|F_o| \times 100$, where $F_o$ is the observed structure factor and $F_c$ is the calculated structure factor.

d $R_{\text{free}}$ was calculated from 10% of reflections randomly selected, as defined by the CNS (23).
Table II D88N/ΔGlcA-GalNAc and UGL interaction at the active site

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<th>Sugar</th>
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<th>Protein</th>
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<th>Atom</th>
<th>Protein</th>
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<th>Atom</th>
<th>Protein</th>
<th>Distance (Å)</th>
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<td>GalNAc</td>
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<tr>
<td></td>
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<td>O3</td>
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<td>N62</td>
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<td>Glu-211</td>
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Table III Kinetic parameters for wild-type and mutant UGLs

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<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{\text{cat}}/K_m$ (µM$^{-1}$ms$^{-1}$)</th>
<th>Relative $k_{\text{cat}}/K_m$ (%)</th>
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<tr>
<td>Wild-type*</td>
<td>7.3 ± 0.3</td>
<td>90 ± 11</td>
<td>81 ± 27</td>
<td>100</td>
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<td>D88N*</td>
<td>0.00057 ± 0.000052</td>
<td>200 ± 38</td>
<td>0.0029 ± 0.014</td>
<td>0.0036</td>
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<td>D149N*</td>
<td>0.0059 ± 0.00018</td>
<td>60 ± 5.8</td>
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<td>R221A</td>
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<td>69 ± 12</td>
<td>61 ± 24</td>
<td>75</td>
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<td>H193A</td>
<td>0.42 ± 0.012</td>
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<td>Q211A</td>
<td>0.20 ± 0.015</td>
<td>170 ± 26</td>
<td>1.1 ± 0.57</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* These values were obtained from a previous report (18).
Structure of UGL complexed with substrate

Fig. 1. Itoh et al.

(a) 
(b) 
(c) 
(d)
Structure of UGL complexed with substrate

Fig. 2. Itoh et al.

a

b
Fig. 3. Itoh et al.

Structure of UGL complexed with substrate
Fig. 4. Itoh et al.

Structure of UGL complexed with substrate
**Fig. 5. Itoh et al.**

Structure of UGL complexed with substrate
Crystal structure of unsaturated glucuronyl hydrolase complexed with substrate: Molecular insights into its catalytic reaction mechanism
Takafumi Itoh, Wataru Hashimoto, Bunzo Mikami and Kousaku Murata

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