Crystal Structure of a Bacterial Unsaturated Glucuronoyl Hydrolase with Specificity for Heparin*

Yusuke Nakamichi1, Bunzo Mikami2, Kousaku Murata1, 1, and Wataru Hashimoto2

From the 1Laboratory of Basic and Applied Molecular Biotechnology, Division of Food Science and Biotechnology, Graduate School of Agriculture, and the 2Laboratory of Applied Structural Biology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan

Background: Bacterial unsaturated glucuronoyl hydrolase (UGL) is essential for complete degradation of host glycosaminoglycans.

Results: Crystal structure of Pedobacter heparinus UGL, Phep_2830 specific for heparin degradation, was determined.

Conclusion: The pocket-like structure and lid loop of Phep_2830 are involved in heparin disaccharide recognition.

Significance: This work contributes to understanding the bacterial degradation of host extracellular matrix components.

Extracellular matrix molecules such as glycosaminoglycans (GAGs) are typical targets for some pathogenic bacteria, which allow adherence to host cells. Bacterial polysaccharide lyases depolymerize GAGs in β-elimination reactions, and the resulting unsaturated disaccharides are subsequently degraded to constituent monosaccharides by unsaturated glucuronoyl hydrolases (UGLs). UGL substrates are classified as 1,3- and 1,4-types based on the glycoside bonds. Unsaturated chondroitin and heparin disaccharides are typical members of 1,3- and 1,4-types, respectively. Here we show the reaction modes of bacterial UGLs with unsaturated heparin disaccharides by x-ray crystallography, docking simulation, and site-directed mutagenesis. Although streptococcal and Bacillus UGLs were active on unsaturated heparin disaccharides, those preferred 1,3- rather than 1,4-type substrates. The genome of GAG-degrading Pedobacter heparinus encodes 13 UGLs. Of these, Phep_2830 is known to be specific for unsaturated heparin disaccharides. The crystal structure of Phep_2830 was determined at 1.35-Å resolution. Docking simulations of Phep_2830 with unsaturated disaccharides demonstrated that the direction of substrate pyranose rings differs from that in unsaturated chondroitin disaccharides. Acetyl groups of unsaturated heparin disaccharides are well accommodated in the pocket at subsite +1, and aromatic residues of the lid loop are required for stacking interactions with substrates. Thus, site-directed mutations of the pocket and lid loop led to significantly reduced enzyme activity, suggesting that the pocket-like structure and lid loop are involved in the recognition of 1,4-type substrates by UGls.

Glycosaminoglycan (GAG)3 is a heteropolysaccharide comprising uronic acid and amino sugar residues (1). Based on the sugar composition and mode of glycosidic bonds (i.e. 1,3- and 1,4-types), GAGs are categorized as hyaluronan, chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin (Fig. 1, A and B). The polysaccharide chondroitin sulfate has repeating disaccharide units comprising β-D-glucuronic acid (GlcUA) and N-acetyl-D-galactosamine (GalNAc), which are linked via 1,3-glycosidic bonds. In contrast, heparin and heparan sulfate have only 1,4-type glycosidic bonds, which comprise repeating disaccharide units with uronic acid residues (α-L-iduronic acid (IdoUA) or β-GlcUA) and amino sugar residues (D-glucosamine (GlcN) or N-acetyl-D-glucosamine (GlcNAc)). Constituent sugar residues of chondroitin sulfate and heparin have varying numbers of sulfate groups (2). With the exception of hyaluronan, these GAGs are present as core protein-bound proteoglycans and play multiple roles in the architecture of extracellular matrices and cell growth and differentiation (3).

Adhesion of microbes to eukaryotic cells may be a primary mechanism for residence of normal flora and pathogenic infections. GAGs are typical microbial targets for interactions with host cells, and some specific interactions between microbes and these polysaccharides have been described (4, 5). Such

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This article contains supplemental Fig. S1.

The atomic coordinates and structure factors (code 3WIW) have been deposited in the Protein Data Bank (http://wwwpdb.org).

1 Present address: Faculty of Science and Engineering, Setsunan University, Neyagawa, Osaka 572-8508, Japan.
2 To whom correspondence should be addressed. Tel.: 81-774-38-3756; Fax: 81-774-38-3767; E-mail: whasimot@kais.kyoto-u.ac.jp.

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microbes include GAG-degrading bacteria. GAGs are depolymerized by a variety of bacterial polysaccharide lyases. Based on primary structures, these enzymes are categorized into several families (PL-1–22) in the Carbohydrate-Active enZymes (CAZy) database (6–10). GAG-degrading enzymes include hyaluronate and chondroitin ABC lyases (family PL-8), and heparinase I (PL-13), -II (PL-21), and -III (PL-12), which have differing sequence and substrate specificities (9, 10). Polysaccharide lyases depolymerize GAGs through $\beta$-elimination reactions and generally produce unsaturated disaccharides containing unsaturated uronic acid with C-C double bonds at nonreducing termini (Fig. 1B) (11). Carbon atoms C-3, C-4, C-5, and C-6 of unsaturated GlcUA ($\Delta$GlcUA) and IdoUA ($\Delta$IdoUA) are located in a single plane because of the formation of double bonds between C-4 and C-5 (12). Because GlcUA is a C-5 epimer of IdoUA, $\Delta$GlcUA and $\Delta$IdoUA are identical in structure. Hence, both $\Delta$GlcUA and $\Delta$IdoUA are referred to as $\Delta$GlcUA in this paper.

Unsaturated GAG disaccharides are degraded to monosaccharides by unsaturated glucuronyl hydrolase (UGL), which is classified as a member of the glucoside hydrolase family 88 in the CAZy database (Fig. 1B) (7, 13). In contrast to other hydrolases, UGL recognizes a double bond in $\Delta$GlcUA and triggers the hydration of C-5 (14, 15). Because $\Delta$GlcUA is a component of all unsaturated GAG oligosaccharides, UGLs are essential for the complete degradation of GAGs. To date, all bacterial UGLs are classified as members of the GH-88 family. However, different substrate specificities accommodate structural diversities of unsaturated GAG oligosaccharides with different sugar residues, glycosidic bonds, and degrees of sulfation. For example, streptococcal UGLs and Bacillus sp. GL1 enzyme (BacillusUGL) prefer sulfated and unsulfated unsaturated disaccharides with 1,3-glycosidic bonds, respectively (16, 17). In contrast, Phep_2830, the UGL of Pedobacter heparinus (formerly known as Flavobacterium heparinum) degrades only unsaturated heparin disaccharides with 1,4-glycosidic bonds (18).

Recent studies have focused on the physiological functions and structures of UGLs and have revealed peculiar mechanisms of UGL catalysis using artificial substrates (15) and that UGL gene disruption leads to reduced upper respiratory tract colonization by Streptococcus pneumoniae (19). Specific inhibitors of UGL are therefore expected to provide anti-bacterial drugs with no side effects. In addition, we demonstrated inducible mRNA expression of the streptococcal enzyme in the presence of bacteria.
of GAG (16) and identified structural determinants of the preference of streptococcal UGL for sulfated substrates with 1,3-glycosidic bonds (12). However, the enzyme recognition mechanism for 1,4-glycosidic bond-type substrates from heparin and heparan sulfate remains unknown. Degradation of heparins and heparan sulfates with 1,4-glycosidic bonds is also considered important for bacterial adherence and invasion to host cells because heparin, heparan sulfate, hyaluronan, and chondroitin sulfate are constituents of mammalian extracellular matrices (20). In this study, we examined the crystal structure of a P. heparinus UGL that has specific activity for unsaturated disaccharides with 1,4-glycosidic bonds and demonstrated the binding modes of these substrates with bacterial UGLs by x-ray crystallography, docking simulations, site-directed mutagenesis, and measurements of mutant enzyme kinetics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unsaturated chondroitin disaccharides were purchased from Seikagaku Biobusiness (Tokyo, Japan). Unsaturated heparin disaccharides were purchased from Iduron (Manchester, UK) and Sigma. Restriction endonucleases and DNA-modifying enzymes were purchased from Toyobo (Osaka, Japan). All other analytical grade chemicals were obtained from commercial sources.

**Overexpression**—Overexpression systems for P. heparinus UGLs (Phep_2238, Phep_2649, and Phep_2830) were constructed in Escherichia coli cells as follows. Three P. heparinus UGL genes were amplified from the genome of P. heparinus by polymerase chain reaction (PCR). PCR was performed using P. heparinus genomic DNA as a template, synthetic oligonucleotide primers, and KOD-FX-neo polymerase (Toyobo). Forward and reverse primers for the Phep_2238 gene were 5'-GGCATATAAGAAGAAACCTGATT TTTAAAAA-3’ and 5’-CCCTCGAGTTAAGACTGATTAATTGTTTTC-3’, those for the Phep_2649 gene were 5'-GGCATATGAACTGATTTTCAAGTCAAGTCC-3’ and 5’-CCCTCGAGTTATTTCTGTAGTTTTTTATAACGC-3’; those for the Phep_2830 gene were 5’-GGCATATGAAAAGAAACCTGATTTTAAAAA-3’ and 5’-CCCTCGAGTCATAAACCCCTCGAGTTAAGACTGATTAATTGTTTTC-3’. Restriction sites for Ndel and Xhol are shown as underlined 5’ regions. The PCR conditions were as follows: 94°C for 2 min followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 30 s. PCR products were ligated with HincII-digested pUC-119 (Takara, Shiga, Japan) using Ligation High version 2 (Toyobo) in accordance with the manufacturer’s protocol, and the resulting plasmids were digested with Ndel and Xhol to isolate UGL gene fragments. Phep_2238 and Phep_2649 DNA fragments were ligated with Ndel- and Xhol-digested pTEt21b vectors (Novagen, Darmstadt, Germany), and the Phep_2830 DNA fragment was ligated into Ndel- and Xhol-digested pCold IV vector (Takara Bio, Shiga, Japan) using Ligation High version 2. The resulting plasmids were designated pET21b-Phep_2238, pET21b-Phep_2649, and pCold IV-Phep_2830, respectively.

To overexpress Phep_2238, Phep_2649, and Phep_2830, E. coli BL21(DE3) host cells (Novagen) were transformed with pET21b-Phep_2238 and pET21b-Phep_2649, and E. coli Rosetta-gami B host cells (Novagen) were transformed with pCold IV-Phep_2830.

**Microorganisms and Culture Conditions**—To express Phep_2238 or Phep_2649, E. coli BL21(DE3) cells harboring pET21b-Phep_2238 or pET21b-Phep_2649 plasmid were cultured at 37°C in Luria broth (Sigma) supplemented with sodium ampicillin (100 μg ml⁻¹). When turbidity at 600 nm reached 0.3–0.7, isopropyl β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.1 mM, and the cells were further cultured at 16°C for 44 h. To overexpress Phep_2830, E. coli Rosetta gami B cells harboring pCold IV-Phep_2830 were cultured at 37°C in Luria broth supplemented with sodium ampicillin (100 μg ml⁻¹) and chloramphenicol (33 μg ml⁻¹). When turbidity at 600 nm reached 1–1.2, the cells were cooled to 15°C with ice water, isopropyl β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.4 mM, and the cells were further cultured at 15°C for 44 h.

**Purification**—Purification of Streptococcus agalactiae UGL (SagUGL), S. pneumoniae UGL (SpnUGL), Streptococcus pyogenes UGL (SpyUGL), SagUGL mutant S368G (12), and Bacillus UGL were expressed and purified as described previously (12, 16, 21). E. coli cells harboring pET21b-Phep_2238, pET21b-Phep_2649, or pCold IV-Phep_2830 plasmid were collected by centrifugation at 6700 × g for 10 min at 4°C. Cells harboring pET21b-Phep_2238 or pET21b-Phep_2649 were resuspended in 20 mM potassium phosphate buffer (KPB, pH 7.0), and the cells harboring pCold IV-Phep_2830 were resuspended in a solution containing 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 1 mM EDTA. Cells were ultrasonically disrupted (Insonator model 201 M; Kubota, Osaka, Japan) at 9 kHz for 10 min at 0°C, and the supernatant obtained by centrifugation at 28000 × g for 20 min at 4°C was used as a cell extract. Phep_2238 and Phep_2649 were purified by cation exchange chromatography (TOYOPEARL CM-650, Tosoh, Tokyo, Japan) followed by gel filtration chromatography (HiLoad 16/60 Superdex 75 pg, GE Healthcare). Phep_2830 was purified by anion exchange chromatography (HiLoad 16/10 Q-Sepharose, GE Healthcare) followed by gel filtration chromatography (HiLoad 16/60 Superdex 75 pg). Purity was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22).

**Enzyme Assay**—Reactions of SagUGL, SpnUGL, SpyUGL, and Bacillus UGL were conducted at 30°C in 500-μl solutions of 20 mM Tris-HCl (pH 7.5), 0.2 mM substrate, and enzyme. Reactions of Phep_2238, Phep_2649, and Phep_2830 were conducted at 30°C in 500-μl solutions containing 100 mM KPB (pH 6.0), 0.2 mM substrate, and enzyme. Concentrations and pH of each buffer were adopted in accordance with previous reports (12, 18). Enzyme activity was measured by monitoring decreases in absorbance at 235 nm, which corresponded to the loss of substrate C-C double bonds.

Unsaturated chondroitin disaccharides were used as substrates, and their molar absorption coefficients at 235 nm (ε<sub>235</sub> (m<sup>-1</sup> cm<sup>-1</sup>)) were as follows: ΔGlcUA-1,3-GalNAc (CΔbOS), ε<sub>235</sub> = 4800; ΔGlcUA-1,3-GalNAc with a sulfate group at the C-4 position of GalNAc (CΔ4S), ε<sub>235</sub> = 4800; and ΔGlcUA-1,3-GalNAc with a sulfate group at the C-6 position of GalNAc (CΔ6S), ε<sub>235</sub> = 4800 (12). Unsaturated heparin disaccharides were also used as substrates, and their molar absorption coefficients at 235 nm were as follows: ΔGlcUA-1,4-GlcNAc (HΔNacOS), ε<sub>235</sub> = 4524; ΔGlcUA-1,4-GlcN with a sulfate
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group at the N position of GlcN (H\(\Delta NS\)), \(\epsilon_{235} = 6600\); \(\Delta\text{GlcUA-1,4-GlcN}\) with a sulfate group at the C-6 position of GlcN (H\(\Delta 6S\)), \(\epsilon_{235} = 4826\); \(\Delta\text{GlcUA-1,4-GlcNAC}\) with a sulfate group at the C-6 position of GlcNAC (H\(\Delta N\ac 6S\)), \(\epsilon_{235} = 4300\); \(\Delta\text{GlcUA-1,4-GlcN}\) with sulfate groups at N and C-6 positions of GlcN (H\(\Delta NS 6S\)), \(\epsilon_{235} = 6075\); \(\Delta\text{GlcUA-1,4-GlcN}\) with sulfate groups at the C-2 position of \(\Delta\text{GluCA}\) and the N position of GlcN (H\(\Delta 2\ac SNS\)), \(\epsilon_{235} = 4433\) (18).

Kinetic parameters of SagUGLs (wild-type, S365A, S365G, S368A, S368G, and K370A), BacillusUGL, Phep_2238, Phep_2649, and Phep_2830s contained substrate, 100 mM KPB (pH 6.0), and enzyme. Substrate concentration ranges were 0.05–1.0 mM because the absorbance at 235 nm of the substrate at over 1.0 mM exceeds measurement limitations on the spectrometer. Michaelis-Menten constants (\(K_m\)) and turnover numbers (\(k_{cat}\)) were calculated by fitting the data using the KaleidaGraph software (Synergy Software, Reading, PA).

Docking Simulations—To perform docking analyses of UGLs and substrates, our coordinates of SagUGL were used from the Protein Data Bank (PDB). The structure of ligand-free SagUGL (PDB code 2ZZR) was selected as a receptor model. Docking analyses were performed using the AutoDock 4.2 program (23). The coordinates of C\(\Delta 6S\) were obtained from coordinates of SagUGL complexed with C\(\Delta 6S\) (PDB code 3ANK), and coordinates of unsaturated heparin disaccharides were obtained using ACD/ChemSketch Freeware, version 5.12 (Advanced Chemistry Development, Inc., Toronto, ON, Canada) and the OpenBabel program (24). Asp-175 and Lys-370 of SagUGL were treated as flexible residues because Asp-175 of SagUGL was critical for catalysis and Lys-370 showed movement during interactions with C\(\Delta 6S\) (12, 16). The number of genetic algorithm runs was set to 20. Figures for protein structures and docking forms were prepared using the PyMOL program (25).

Site-directed Mutagenesis—Three residues (Ser-365, Ser-368, and Lys-370) of SagUGL were substituted with Asn, Ala, and Lys, and the resulting mutants were designated S365A, S365G, S368A, and K370A, respectively. Asp-182 of Phep_2238, and Arg-57, Phe-164, Asp-174, and Gly-362 of Phep_2830 were also substituted with Asn, Ala, Asn, and Tyr, and the resulting mutants were designated D182N, R57A, F164A, D174N, and G362Y, respectively. With the exception of D174N and G362Y, UGL mutants were constructed using a QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). D174N and G362Y were constructed using a KOD-Plus Mutagenesis Kit (Toyobo). Mutations were confirmed using the dye-dye chain termination method with the automated DNA sequencer model 3730xl (Applied Biosystems) (26). E. coli HMS174(DE3) host cells (Novagen) were transformed with SagUGL mutant plasmids. Expression and purification of SagUGL mutants were conducted using the procedure for wild-type SagUGL as described previously (12). Phep_2830 mutant plasmids were digested with NdeI and XhoI, and the resulting DNA fragments were ligated with NdeI and XhoI-digested pCold II vector (Takara Bio) to express Phep_2830 mutant proteins with N-terminal His tags for purification. N-terminal His-tagged Phep_2830 reportedly exhibits comparable enzyme activity to that of the His tag-free enzyme (18). The Phep_2830 mutants R57A, F164A, and G362Y were purified to almost homogeneity by affinity chromatography (TALON, Clontech).

X-ray Crystallography—To determine the three-dimensional structure of Phep_2830, the purified enzyme was crystalized using sitting drop vapor diffusion. Solutions containing 1 \(\mu\)l of proteins (10 mg ml\(^{-1}\)) in 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 1 mM EDTA were mixed with equal volumes of reservoir solution containing 25% polyethylene glycol 6000, 5% ethylene glycol, and 0.1M HEPES (pH 7.5), and enzyme. Reaction mixtures for Phep_2238, Phep_2649, and Phep_2830s contained substrate, 100 mM KPB (pH 6.0), and enzyme. Substrate concentration ranges were 0.05–1.0 mM because the absorbance at 235 nm of the substrate at over 1.0 mM exceeds measurement limitations on the spectrometer. Michaelis-Menten constants (\(K_m\)) and turnover numbers (\(k_{cat}\)) were calculated by fitting the data using the KaleidaGraph software (Synergy Software, Reading, PA).

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tioning of protein models and calculation of their root mean square deviations were conducted using the LSQKAB program supplied with the CCP4 program package (34).

RESULTS AND DISCUSSION

Enzyme Activity of Streptococcal UGLs toward Unsaturated Heparin Disaccharides—Three streptococcal UGLs (SagUGL, SpnUGL, and SpyUGL) were purified to homogeneity, and their enzyme activities (units mg$^{-1}$) toward a variety of unsaturated heparin disaccharides were measured (Table 1). All three streptococcal UGLs degraded H$_2$H$_9$NS with greater efficiency than the other unsaturated heparin disaccharides. Kinetic parameters of SagUGL toward H$_2$H$_9$NAc$_6$S were determined as follows: $K_m$, 1.9 mM; $k_{cat}$, 3.9 s$^{-1}$. SagUGL is known to exhibit the highest enzymatic activity toward C$_6$H$_9$O$_6$ with 1,3-glycosidic bond (12) as follows: $K_m$, 0.10 mM; $k_{cat}$, 10 s$^{-1}$, indicating that the enzyme activity of SagUGL toward unsaturated heparin disaccharides was lower than that toward unsaturated con-

![FIGURE 2. Binding mode of unsaturated disaccharides to bacterial UGLs (stereo diagram). A, our crystal structure of SagUGL in complex with C$_6$H$_9$S determined by x-ray crystallography (PDB code 3ANK); B, an active site structure of SagUGL/C$_6$H$_9$S; C, an active site structure of SagUGL/H$_2$H$_9$NS; D, an active site structure of Phep$_{2830}$/H$_2$H$_9$NS; E, surface of the pocket recognizing acetyl group; B–E, structures estimated via docking simulations; numbers of carbon atoms are shown in B and C. Atoms C of SagUGL, C of Phep$_{2830}$, O, N, and S are in yellow, purple, red, blue, and green, respectively.]

| Substrate specificity of bacterial UGLs | Specific activity |
|----------------------------------------|------------------|
| SagUGL | SpnUGL | SpyUGL | BacillusUGL | Phep$_{2238}$ | Phep$_{2649}$ | Phep$_{2830}$ |
| H$_2$H$_9$NAc$_6$S | 0.016 | 0.0055 | 0.0033 | 5.3 | 12 | 0.30 | 15 |
| H$_2$H$_9$NS | 0.77 | 0.14 | 0.10 | 1.8 | 11 | 1.1 | 7.1 |
| H$_2$H$_9$S | 0.0088 | 0.0018 | 0.00094 | 0.081 | 1.2 | ND* | 1.9 |
| H$_2$H$_9$NAc$_6$S | 0.035 | 0.0059 | 0.0066 | 0.016 | 7.9 | ND | 18 |
| H$_2$H$_9$S$_6$ | 0.0080 | 0.0031 | ND | 0.0050 | 4.2 | ND | 4.5 |
| C$_6$H$_9$S | 0.0023 | 0.0024 | ND | 0.0095 | ND | ND | ND |
| C$_6$H$_9$S$_6$ | 0.72 | 0.99 | 0.63 | 10 | 2.6 | 0.70 | ND |
| C$_6$H$_9$O$_6$ | 0.0019 | ND | 0.0046 | ND | 0.31 | ND | ND |
| C$_6$H$_9$O$_6$$_6$ | 17 | 6.1 | 5.3 | 1.8 | 3.2 | 1.7 | ND |

* Not detected.
droitin disaccharides. Indeed, \( k_{\text{cat}} / K_m \) with H\( \text{HANS} \) was 50-fold lower than with C\( \text{A6S} \) (12). The enzyme activities of BacillusUGL with each unsaturated heparin disaccharide were also measured. BacillusUGL exhibited the highest enzyme activity toward H\( \text{HANS} \)AcOS distinct from streptococcal UGLs (Table 1). BacillusUGL also demonstrated specificity for unsulfated unsaturated chondroitin disaccharides (17). These results and our previous reports (16, 17) demonstrate that streptococcal UGLs acted on unsaturated heparin disaccharides, whereas BacillusUGL preferentially degrades unsulfated substrates.

**Table 2**: Kinetic parameters of SagUGL mutants for H\( \text{HANS} \)

| Mutant | \( K_m \) (mM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}} / K_m \) (s\(^{-1}\) mM\(^{-1}\)) |
|--------|----------------|-----------------|-----------------|
| WT     | 1.9 ± 0.2      | 3.8 ± 0.4       | 2.0 ± 0.3       |
| S365A  | 3.4 ± 1.3      | 1.9 ± 0.6       | 0.56 ± 0.14     |
| S365G  | 5.2 ± 2.7      | 0.76 ± 0.35     | 0.15 ± 0.07     |
| S368A  | 3.0 ± 1.0      | 6.0 ± 1.7       | 2.0 ± 0.4       |
| S368G  | 3.4 ± 0.7      | 2.8 ± 0.5       | 0.83 ± 0.05     |
| K370A  | 0.86 ± 0.29    | 0.075 ± 0.014   | 0.086 ± 0.013   |

**Table 3**: Data collection and refinement statistics

| Parameter                         | Value          |
|-----------------------------------|----------------|
| Unit cell parameters (Å, deg)     |                |
| a                                 | 91.84          |
| b                                 | 110.00         |
| c                                 | 110.00         |
| Resolution limit (Å)              | 1.35 (1.38–1.35) |
| R-factor (%)                      | 13.2 (14.9)    |
| R\( \text{free} \) (%)            | 16.0 (21.4)    |
| Protein                           | 32.44          |
| HEPES                             | 15             |
| H\( \text{O} \)                    | 422            |
| Average isotropic B-factor (Å\(^{2}\)) | 16.7          |
| Protein                           | 30.1           |
| H\( \text{O} \)                    | 27.5           |
| Root mean square deviation from ideal |                |
| Bond length (Å)                   | 0.009          |
| Bond angle (deg)                  | 1.226          |
| Ramachandran plot (%)             | 97.8           |
| Favored region (%)                | 2.2            |
| Allowed region (%)                | 0.013          |

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| R\( \text{free} \) (%)            | 16.0 (21.4)    |
| Final model                        |                |
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Data in highest resolution shells are given in parentheses.
rinus, which assimilates heparin as a carbon source, is known to specifically degrade substrates containing 1,4-glycosidic bonds. Thirteen UGL genes of P. heparinus were assigned by complete genome sequences (36) and the three UGLs Phep_2238, Phep_2649, and Phep_2830 were overexpressed in E. coli cells and purified to homogeneity and assayed for activity (Table 1).

Phep_2238 degraded HβNAc0S and HαNS more efficiently than other unsaturated heparin disaccharides. Moreover, Phep_2238 exhibited comparably lower enzyme activity toward substrates with 1,3-glycosidic bonds, such as Cα0S, Cα4S, and Cα6S, than those with 1,4-glycosidic bonds. The kinetic parameters $K_m$ and $k_{cat}$ of Phep_2238 toward HαNS were 0.073 mM and 2.3 s⁻¹, respectively. Phep_2649 preferred Cα6S but also
degraded C\(_\Delta\)OS, H\(_\Delta\)NAc0S, and H\(_\Delta\)NS, although its specific activity was lower than that of the streptococcal UGLs, Bacillus-UGL, Phep_2238, and Phep_2830. \(K_m\) and \(k_{cat}\) values of Phep_2649 toward H\(_\Delta\)NS were 0.052 mM and 0.17 s\(^{-1}\), respectively. Although a UGL that degrades unsaturated chondroitin disaccharide (C\(_6\)A6S) was previously isolated as a 1,3-glycuronidase from \(P.\) heparinus (37), this enzyme differs from Phep_2238 and Phep_2649 in amino acid composition, isoelectric point, and molecular weight. In contrast, Phep_2830 exhibited enzyme activity toward only unsaturated heparin disaccharides, as described previously (18). \(K_m\) and \(k_{cat}\) values of Phep_2830 toward H\(_\Delta\)NAc6S were 0.029 mM and 16 s\(^{-1}\), respectively.

Crystal Structure of Phep_2830—Because Phep_2238 and Phep_2830 showed high enzyme activity toward various unsaturated heparin disaccharides, these enzymes were crystallized to clarify the mechanisms by which UGL recognizes substrates with 1,4-glycosidic bonds based on tertiary structures. Phep_2830 was successfully crystallized, and x-ray diffraction data were collected. The crystal structure of Phep_2830 was determined at a resolution of 1.35 Å using molecular replacement with the SagUGL structure (PDB code 2ZZR) as an initial model. Data collection and model refinement statistics are summarized in Table 3. The final model contains one monomer enzyme from Gly-28 to Thr-398, a molecule of HEPES, and 422 water molecules. The N-terminal region from Met-1 to Asn-27 could not be assigned because the electron density map was too thin. Similar to SagUGL and BacillusUGL, the overall structure of Phep_2830 has a \(\alpha\)/\(\beta\)-barrel architecture, and Phep_2830 contains 12 \(\alpha\) helices and 5 \(\beta\) strands (Fig. 3A). The root mean square deviation for all of the 336 \(C^\alpha\) atoms between Phep_2830 and SagUGL was 1.7 Å, indicating that both have a common basic scaffold structure. In contrast, \(\sim 10\) C-terminal amino acid residues of Phep_2830 protrude forward from the outside of the protein. Previous studies show that Asp-149 of BacillusUGL and Asp-175 of SagUGL act as critical catalysts (14, 16). Thus, to investigate the catalytic mechanisms of \(P.\) heparinus UGLs, corresponding residues of Phep_2238 (Asp-182) and Phep_2830 (Asp-174) were substituted with Asn. Both mutant enzymes were inactive, indicating that the catalytic mechanisms of Phep_2238 and Phep_2830 are similar to those of SagUGL and BacillusUGL.

Active sites were structurally compared by superimposing coordinates of Phep_2830 on those of SagUGL (PDB code 2ZZR) and BacillusUGL/C\(_4\)OS (PDB code 2AHG; Fig. 3, B–D). Subsites were defined such that \(- n\) represents the nonreducing terminus, \(+ n\) represents the reducing terminus, and cleavage occurs between these sites (38). The amino acid residues and their positions at subsite \(- 1\), which is the binding site of \(\Delta\)GlcUA, were common to all three UGLs (Fig. 3B). However, the structures at subsite \(+ 1\), which is the binding site of an amino sugar, differed significantly (Fig. 3, C and D). In particular, Ser-365, Ser-368, and Lys-370 of SagUGL comprise the motif SXXXSK, which contributes to the recognition of a sulfate group (12). These three residues are not conserved in Phep_2830 and correspond to the Ala-363, Tyr-366, and Ser-368 residues of Phep_2830, respectively. These observations indicate that Phep_2830 recognition of substrate sulfate groups differs from that of SagUGL. Regarding hydrophobic amino acid residues forming a stacking interaction with an amino sugar at the subsite \(+ 1\), the Tyr-338 residue of BacillusUGL (or the Tyr-364 residue of SagUGL), which is located around the C-6 position of GalNAc in BacillusUGL/C\(_4\)OS is substituted with the Gly-362 residue in Phep_2830, whereas the Trp-134 residue of BacillusUGL (or the Trp-161 residue of SagUGL) is conserved in Phep_2830. Due to this difference, this large space in Phep_2830 occurs around Gly-362, and is distinct from that of SagUGL and BacillusUGL. As described above, the Phe-164 residue of Phep_2830 that corresponds to Glu-163 of SagUGL or Pro-136 of BacillusUGL also lies at the binding site of an amino sugar (Fig. 3, C and D). The loop comprising amino acid residues 163–169 of Phep_2830, which is designated loop A and corresponds to residues 162–170 of SagUGL and 135–144 of BacillusUGL, covers the active site. However, these loops of both SagUGL and BacillusUGL are distal from those at the active site. Accordingly, the Phe-164 residue of Phep_2830 is proximal to the active site (Fig. 3D). These Phep_2830-specific amino acid residues may be involved in stacking interactions with substrates, especially with amino sugars. Indeed, whereas Arg-57, Arg-66, and Glu-369 residues of Phep_2830 are arranged at subsite \(+ 1\), these are not conserved in the active sites of either SagUGL or BacillusUGL.

Binding Mode of Unsaturated Heparin Disaccharides to Phep_2830—To clarify the mechanism by which Phep_2830 recognizes unsaturated heparin disaccharides, we attempted to prepare complexes of Phep_2830 with these substrates but failed. Thus, the binding modes of unsaturated heparin disaccharides (H\(_\Delta\)Nac0S, H\(_\Delta\)NS, and H\(_\Delta\)Nac6S) to Phep_2830 were estimated in a similar way to those of SagUGL using the AutoDock program. In these simulations, structures of H\(_\Delta\)Nac0S, H\(_\Delta\)NS, and H\(_\Delta\)Nac6S-bound Phep_2830 were successfully calculated and that of the Phep_2830-H\(_\Delta\)Nac0S complex is shown in Fig. 2D. Two common features were observed in the three docking structures that exhibited low binding energies. Similar to the docking simulation of SagUGL with H\(_\Delta\)NS,
the direction of the pyranose ring of GlcNAc (or GlcN sulfated at the N position) toward ΔGlcUA was opposed to that of Gal-NAc in complex with SagUGL/C_H9004_6S and BacillusUGL/C_H9004_0S (Fig. 1C). In addition, the acetyl groups of HNAc0S and HNAc6S (or the sulfate group of HNS) were predicted to be accommodated in the pocket-like structure comprising Arg-57, Arg-66, Trp-73, Gly-362, Ala-363, Tyr-366, Ser-368, and Glu-369 residues, which was designated the acetyl/sulfate group-binding pocket (Fig. 2, D and E). No similar pocket-like structures were observed in SagUGL and BacillusUGL, which prefer substrates with 1,3-glycosidic bonds (Fig. 3C). The Trp-162 and Phe-164 residues in loop A of Phep_2830 (Fig. 3D) are located close to the C-6 position of an amino sugar of unsaturated heparin disaccharides. As described above, Trp-161, Trp-134, and Trp-162 residues of SagUGL, BacillusUGL, and Phep_2830, respectively, are situated at almost identical positions, whereas the residues of SagUGL and BacillusUGL that correspond to Phe-164 of Phep_2830 are distal from subsite 1.

To confirm that this pocket-like structure and loop A contribute to recognition of unsaturated heparin disaccharides, Arg-57, Phe-164, and Gly-362 residues of Phep_2830 were substituted with Ala, Ala, and Tyr, respectively. The resulting mutants R57A, F164A, and G362Y with N-terminal His tags were expressed in E. coli and were purified using affinity columns. Although their expression was confirmed by SDS-PAGE (Fig. 4), two mutants of the pocket-like structure (R57A and G362Y) exhibited no detectable enzyme activity with unsaturated heparin disaccharides as well as unsaturated chondroitin.
disaccharides. The $K_m$ value (1.4 mM) of F164A toward HΔNAc6S was about 50-fold higher than that (0.029 mM) of the wild-type enzyme, whereas there was no significant difference in $k_{cat}$ between wild-type enzyme (16 s$^{-1}$) and F164A (22 s$^{-1}$). These site-directed mutagenesis experiments indicated that the pocket-like structure and loop A play a significant role in recognizing substrates. In contrast, the absence of the acetyl group may have led to low specific activity of Phep_2830 toward HΔ6S and hampered docking simulations of the complex of Phep_2830 with HΔ6S. Hence, substrates containing 1,4-glycosidic bonds are readily inserted into the acetyl/sulfate group-binding pocket of Phep_2830 through stacking interactions of loop A residues, Trp-162 and Phe-164, with an amino sugar. Simultaneously, Trp-162 and Phe-164 residues may inhibit binding of Phep_2830 with substrates containing 1,3-glycosidic bonds.

Comparisons of the active site structures and docking simulations suggest that the acetyl/sulfate group-binding pocket comprising Arg-57, Arg-66, Trp-73, Gly-362, Ala-363, Ser-368, and Glu-369, and the loop A including Trp-162 and Phe-164, are important for binding of Phep_2830 to unsaturated heparin disaccharides. Among these amino acid residues, Arg-57, Trp-73, Gly-362, Glu-369, and Trp-162 are conserved in Phep_2238 and Phep_2649 (Fig. 5). Moreover, Arg-66, Ala-363, Tyr-366, Ser-368, and Phe-164 of Phep_2830 correspond with Val-73, His-373, Gly-376, Ser-378, and Ser-171 of Phep_2238 and Arg-59, Ser-362, His-365, Asn-367, and Val-158 of Phep_2649, respectively. These differences in primary structure may lead to changes in pocket shapes but do not occupy the space. However, Ser-171 of Phep_2238 and Val-158 of Phep_2649, which correspond to Phe-164 of Phep_2830, do not form stacking interactions because they are smaller than Phe. Hence, these structural features may enable recognition of substrates with 1,3-glycosidic bonds by Phep_2238 and Phep_2649. Although the pocket-like structure of Phep_2649 was arranged with high probability, the activity of the enzyme for unsaturated heparin disaccharides was lower than that of Phep_2238 and Phep_2830. In addition, the relative length of amino acid residues of Phep_2649 that correspond to loop A may contribute to low enzyme activity.

Bacteroides species, human intestinal bacteria, are known to produce family PL-12, -13, and -21 heparinases depolymerizing heparin to unsaturated disaccharides through a $\beta$-elimination reaction (39–43), whereas complete degradation of heparin to monosaccharides remains to be clarified. Four UGL-homologous proteins are encoded in the genomes of Bacteroides stercoris (BACSTE_02470, BACSTE_03202, BACSTE_03210, and BACSTE_03707) and Bacteroides thetaiotaomicron (BT_0146, BT_2913, BT_3348, and BT_4658). Arg-57 and Gly-362, key residues comprising the pocket-like structure of Phep_2830, are conserved in these bacteroides UGLs (supplemental Fig. S1), suggesting that, similar to Phep_2238 and Phep_2830, bacteroides enzymes preferentially degrade unsaturated heparin disaccharides to constituent monosaccharides. Consequently, structural determinants of Phep_2830 for 1,4-specificity found in this study promotes a better understanding of the complete degradation of heparin by the Bacteroides species.

In conclusion, tertiary and active site structures of UGLs that are specific for unsaturated heparin disaccharides containing 1,4-glycosidic bonds were determined for the first time. Comparisons of the active site structures, docking simulations, and site-directed mutagenesis experiments demonstrate the significance of the acetyl/sulfate group-binding pocket and the lid loop at subsite +1 in Phep_2830 for recognition of substrates with 1,4-glycosidic bonds.

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