Crystall Structure and Characterization of the Glycoside Hydrolase Family 62 α-L-Arabinofuranosidase from Streptomyces coelicolor*

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Background: Glycoside hydrolase family 62 α-L-arabinofuranosidases specifically release l-arabinose from arabinoxylan.

Results: The crystal structure of glycoside hydrolase family 62 α-L-arabinofuranosidase from Streptomyces coelicolor was determined.

Conclusion: L-Arabinose and xylohexaose complexed structures revealed the mechanism of substrate specificity of this enzyme.

Significance: Efficient catalysis by glycoside hydrolase family 62 α-L-arabinofuranosidase requires its binding to terminal xylose sugars at the substrate-binding cleft.

α-L-Arabinofuranosidase, which belongs to the glycoside hydrolase family 62 (GH62), hydrolyzes arabinoxylan but not arabinan or arabinogalactan. The crystal structures of several α-L-arabinofuranosidases have been determined, although the structures, catalytic mechanisms, and substrate specificities of GH62 enzymes remain unclear. To evaluate the substrate specificity of a GH62 enzyme, we determined the crystal structure of α-L-arabinofuranosidase, which comprises a carbohydrate-binding module family 13 domain at its N terminus and a catalytic domain at its C terminus, from Streptomyces coelicolor. The catalytic domain was a five-bladed β-propeller consisting of five radially oriented anti-parallel β-sheets. Sugar complex structures with l-arabinose, xylotriose, and xylohexaose revealed five subsites in the catalytic cleft and an L-arabinofuranosyl-potting at the bottom of the cleft. The entire structure of this GH62 family enzyme was very similar to that of glycoside hydrolase 43 family enzymes, and the catalytically important acidic residues found in family 43 enzymes were conserved in GH62. Mutagenesis studies revealed that Asp202a and Glu361 were catalytic residues, and Trp277, Tyr461, and Asn462 were involved in the substrate-binding site for discriminating the substrate structures. In particular, hydrogen bonding between Asn462 and xylose at the nonreducing end subsite +2 was important for the higher activity of substituted arabinoaranosyl residues than that for terminal arabinofuranoses.

Although the content of L-arabinose (Ara)4 in the plant cell walls is low, Ara residues are frequently found in plant cell walls as arabinoxylan, arabinan, arabinogalactan, and others. Because the structures of L-arabinose-containing polysaccharides are highly variable and complex, various α-L-arabinofuranosidases (EC 3.2.1.55) with varying substrate specificities are necessary to hydrolyze these polysaccharides (4). We previously purified some α-L-arabinofuranosidases and elucidated their substrate specificities toward structurally defined substrates (5–16). However, the relationships between the amino acid sequences of these enzymes and their substrate specificities remain to be resolved.

Glycoside hydrolases (GHs) are currently classified into 133 families based on their amino acid sequence similarities, implying that there are both structural and mechanistic relationships (17, 18). α-L-Arabinofuranosidases are divided into five GH families as follows: GH3, -43, -51, -54, and -62. The crystal structures of GH43, -51, and -54 family enzymes have been resolved; however, those of GH3 and GH62 enzymes are not resolved. Thus, the substrate specificity and catalytic mechanism of these enzyme families are poorly understood. It is known that GH62 enzymes belong to the clan GH-F together with GH43 enzymes and are thought to operate an inversion mechanism, as demonstrated in GH43 enzymes. To date, the crystal structures of 24 GH43 enzymes have been resolved (16, 19–28), and the inversion catalytic mechanism was proposed for some enzymes based on their crystal structures and mutagenesis studies (16, 20, 22, 27, 29).

Here, we present the three-dimensional structure of α-L-arabinofuranosidase from Streptomyces coelicolor (ScaAraf62A) and the results of mutagenesis studies based on the structures

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8 The abbreviations used are: Ara, l-arabinose; GH, glycoside hydrolase; GH62, glycoside hydrolase family 62; PNP-l-Araf, p-nitrophenyl-l-arabinofuranoside; ScaAraf62A, Streptomyces coelicolor α-L-arabinofuranosidase, Xy, xylotriose; Xy₃, xylohexaose; A₅, O-α-L-arabinofuranosyl(1←3)-O-β-D-xylopyranosyl(1←4)-D-xylopyranosyl; A₅p, O-β-D-xylopyranosyl(1←4)-O-α-L-arabinofuranosyl(1←3)-O-β-D-xylopyranosyl(1←4)-D-xylopyranosyl.
**EXPERIMENTAL PROCEDURES**

**Expression of ScAraf62A and Mutant Generation**—The primers used in this study are listed in Table 1. ScAraf62A was expressed as a mature protein using the *Streptomyces lividans* expression system (30). The gene encoding the putative α-L-arabinofuranosidase from *S. coelicolor* A3(2) (SC05932; GenBank™ accession number CAA16189) (31) was amplified from *S. coelicolor* genomic DNA by PCR and cloned into a *S. lividans* expression vector (30). Sco5932 (ScAraf62A) has a signal sequence at its N terminus, and the mature protein can be secreted by a cell. The strategy to construct ScAraf62A mutants was as follows; we used site-directed mutagenesis to generate amino acid substitutions in ScAraf62A. Site-directed mutagenesis was performed by PCR with KOD-plus-neo polymerase (Toyobo, Osaka, Japan) using the primer pairs shown in Table 1. Each constructed plasmid was transformed into *S. lividans* 1326, which was used as the host strain. Recombinant ScAraf62A or each of its mutants was secreted into the culture medium and purified by affinity chromatography using lactosyl-Sepharose as described previously (32). The purity of each protein was confirmed by SDS-PAGE.

**Substrates**—p-Nitrophenyl-α-L-arabinofuranoside (PNP-α-L-Araf) , arabinogalactan from larch wood, xylan from oats, and gum arabic as substrates. ScAraf62A was mixed with 0.5% (w/v) branched arabinan, arabinogalactan from larch wood, and gum arabic as substrates. ScAraf62A was mixed with 0.5% (w/v) branched arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, xylotriose (X₃), xylohexaose (X₆), and wheat xylan from birchwood were purchased from Sigma. Debarked arabinan, arabinogalactan from larch wood, xylan from oat spelt, xylan from birchwood, arabinan, debarked arabinan, arabinogalactan from larch wood, and gum arabic as substrates. ScAraf62A was mixed with 0.5% (w/v) substrates in a buffer, pH 6, containing 0.1% (w/v) bovine serum albumin (BSA) and incubated at 45 °C for up to 20 min. The reaction was stopped by heating at 100 °C for 20 min. Hydrolytic activity was determined on the basis of the amounts of reducing sugars as determined by the Somogyi-Nelson method (35).

**ScAraf62A Crystallization**—The purified protein solution was dialyzed against 2 mM Tris-HCl buffer, pH 7.0, containing 20 mM NaCl, concentrated to 8.3 mg ml⁻¹ (A₂₈₀, 1 mm = 2.0 units) by ultrafiltration using a YM-30 membrane (Merck-Millipore), and filtered through a 0.1-μm membrane (Merck-Millipore). The protein was crystallized by the sitting-drop vapor-diffusion method at 293 K using a precipitant solution consisting of 20% (w/v) PEG3350 and 0.2 M tripotassium citrate, pH 8.3. Rod-shaped crystals with maximum dimensions of 0.5 × 0.2 × 0.1 mm were obtained using 50 μl of reservoir solution with a drop consisting of 1.2 μl of protein solution and 1.0 μl of reservoir solution.

**Data Acquisition and Structure Determination**—Diffraction experiments for native and mercury-derivative crystals were performed at the beamline NE3A of the Photon Factory, High Energy Accelerator Research Organization, Tsukuba, Japan. Crystals were mounted on a quartz glass capillary (diameter, 0.3 mm) and then flash-cooled under a nitrogen stream at 95 K. Diffraction data were collected at a wavelength of 1.0031 Å with a Quantum 270 CCD detector (Area Detector Systems Corp., Poway, CA). An mercury-derivative crystal was prepared by adding 0.2 μl of 10 mM ethyl mercuric phosphate to the crystal drop and incubated for 10 min. For structural analyses of the enzyme, the crystal was soaked for 1 drop of a drop containing 5% (w/v) carbohydrates in the precipitant solution for 10 min before the diffraction experiments. Diffraction data for the sugar complexes were acquired at the Photon Factory beamline NW12A (36) or BL-5A. Diffraction data were collected with a Quantum 210 CCD detector (Area Detector Systems Corp.). All data were integrated and scaled using the programs DENZO and SCALEPACK in the HKL2000 program suite (37).

The crystal structure was determined by the single wavelength anomalous dispersion method using a mercury-derivative crystal. Three mercury atom positions were determined, and the initial phases were calculated using the program of the enzymes-Ara and -xylooligosaccharides complexes. These results clearly demonstrate the substrate specificity mechanism of GH62 α-L-arabinofuranosidase and should facilitate further studies on GH62 enzymes.

### Table 1

| Primer  | Nucleotide sequence                                     |
|---------|---------------------------------------------------------|
| Ara6F2A | F, 5‘-CATATGACACGAGAGAAATGCCCAGGGG3’                   |
| D165N   | R, 5‘-GTCCTGGATACCAAACTGGGCCACA3’                       |
| D165E   | R, 5‘-AAGCTTCTAGGCGCTAGCTATGCAG3’                       |
| W233A   | F, 5‘-CTGGGCTTCGCGCCTGCTTCAAAT3’                         |
| W233Y   | R, 5‘-CAGAAGCCGCCTGCTTCAAATCCGACA3’                      |
| W323F   | F, 5‘-CTGGGCTTCGCGCCTGCTTCAAAT3’                         |
| E324Q   | F, 5‘-ACCTGGACCCCTGCAAAATTCCGCTTCAAAT3’                  |
| Y424A   | F, 5‘-GCGGCGGAAAGCCATGCCATGCTTCAAAT3’                    |
| Y424W   | R, 5‘-GCGGCGGAAAGCCATGCCATGCTTCAAATCCGACA3’              |
| Y424F   | F, 5‘-GCGGCGGAAAGCCATGCCATGCTTCAAAT3’                    |
| N425Q   | R, 5‘-GCGGCGGAAAGCCATGCCATGCTTCAAATCCGACA3’              |

* The underlined sequences represent mutation sites.
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The initial model building was conducted by the auto-modeling program ARP/wARP (40) incorporated in the CCP4 program suite (41). Manual model building and molecular refinement were performed using COOT (42) and REFMACS (43, 44).

To analyze sugar complex structures, structural determinations were performed using the ligand-free structure as the starting model, and the bound sugars were observed in the difference electron density map. Data acquisition and refinement statistics are shown in Table 3. The model stereochemistry was determined with the program RAMPAGE (45). Structural drawings were prepared using the program PyMOL (DeLano Scientific LLC, Palo Alto, CA).

**RESULTS**

ScArAraf62A Expression and Purification—The DNA sequence of the *S. coelicolor* Sco5932 gene was 1,428-bp long, and the gene putatively encoded a 475-amino acid protein comprising N-terminal carbohydrate-binding module family 13 (CBM13) and C-terminal GH62 domains. The deduced amino acid sequence was compared with sequences in the protein database using a BLAST search (National Center for Biotechnology Information, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The ScAraf62A (Sco5932) sequence resembled that of AbfB, an α-L-arabinofuranosidase from *S. lividans* (46), and the amino acid similarity was 97% (data not shown). To express ScAraf62A, the DNA fragment encoding the full-length protein was cloned. Recombinant ScArAraf62A was successfully expressed in the secreted form in *S. lividans* and purified as a single band with an apparent molecular size of 44 kDa on SDS-PAGE (data not shown).

ScArAraf62A Characterization and Substrate Specificities—The effects of pH and temperature on ScArAraf62A activity and stability were determined using PNP-α-L-Araf as the substrate (Fig. 1). Maximal enzyme activity was detected at pH 5.5 at 35 °C. ScArAraf62A was stable between pH 7.0 and 9.0 at 35 °C for 1 h. It was also stable up to 30 °C at pH 5.5 for 1 h. The optimum temperature for ScArAraf62A was lower than that for AbfB α-L-arabinofuranosidase from *S. lividans* (55 °C) (46). However, these could not be simply compared because the experimental conditions, such as the substrates used, were different.

Next, we examined the activities of ScArAraf62A for arabinose-containing polysaccharides. ScArAraf62A released Ara from wheat arabinoxylan, arabinoylran from corn hull, and xylan from oat spelt but not from arabinan or arabinogalactan (data not shown). The substrate specificity of ScArAraf62A was similar to that of other GH62 enzymes from *S. lividans*, *Penicillium chrysogenum*, and *Pseudomonas cellulosa* (46–49).

The kinetic parameters of ScArAraf62A for PNP-α-L-Araf and wheat arabinoxylan were determined (Table 2). The *k*₅₀/*Kₘ* value for wheat arabinoxylan was ~4-fold higher than that for PNP-α-L-Araf, suggesting that ScArAraf62A specifically hydrolyzed arabinoylran.

It has been reported that arabinoxylan comprises a β-1,4-linked-xylan backbone that is partially substituted with 2- or 3- or 2,3-linked 1-arabinofuranoses (50). In contrast, arabinan comprises an α-1,5-linked 1-arabinofuranose backbone that is substituted with α-1,3-1-arabinofuranose and/or α-1,2,1-arabinofuranose side chains (51). Interestingly, ScArAraf62A barely reacted with arabinan and possibly reacted with arabinoxylan, although both arabinoxylan and arabinan possess α-1,3-linked 1-arabinofuranose. To evaluate the mechanisms of substrate specificity, we analyzed the crystal structure of ScArAraf62A.

![FIGURE 1. Enzymatic properties of wild-type α-L-arabinofuranosidase from *S. coelicolor* (ScArAraf62A). A, optimum pH; B, optimum temperature; C, stability for pH; D, stability for temperature. Symbols used are as follows: black triangle, glycine-HCl buffer; black circle, McIlvaine buffer; black square, Atkins-Pantin buffer.](image)

**TABLE 2. Activity of wild type and mutants of α-L-arabinofuranosidase from *S. coelicolor* (ScArAraf62A) against PNP-α-L-Araf and wheat arabinoxylan**

The kinetic parameters *k*₅₀ and *K*ₘ were determined by Lineweaver–Burk plots from three independent experiments and with five substrate concentrations. ND, no activity detected; NA, not analyzed.

| Enzyme | PNP-α-L-arabinofuranoside | Wheat arabinoxylan |
|--------|---------------------------|--------------------|
|        | *K*ₘ (mg/ml⁻¹) | *k*₅₀ (min⁻¹) | *k*₅₀/*K*ₘ (min⁻¹) | Relative to WT | *K*ₘ (mg/ml⁻¹) | *k*₅₀ (min⁻¹) | *k*₅₀/*K*ₘ (min⁻¹) | Relative to WT |
| Wild type | 1.9 ± 0.2 | 1.2 ± 0.1 | 0.61 | 1 | 7.3 ± 0.0 | 18 ± 2 | 2.5 | 1 |
| D2D1Q | ND | ND | ND | ND | 27 ± 9 | 16 ± 2 | 0.65 | 0.3 |
| E361Q | ND | ND | ND | ND | 26 ± 3 | 16 ± 3 | 1.2 | 0.5 |
| W270A | 18 ± 1 | 0.71 ± 0.13 | 0.038 | 0.06 | 36 ± 8 | 25 ± 6 | 0.72 | 0.3 |
| W270Y | 11 ± 2 | 0.69 ± 0.08 | 0.068 | 0.1 | 15 ± 4 | 30 ± 9 | 2.0 | 0.8 |
| Y461A | ND | ND | ND | ND | 7.0 ± 0.6 | 3.0 ± 1.05 | 0.43 | 0.2 |
| Y461W | 1.5E⁻¹⁵ ± 0.0 | 7.2E⁻¹¹ ± 0.0 | 0.47E⁻³ | 0.8E⁻³ | 28 ± 13 | 51 ± 23 | 1.8 | 0.7 |
| Y461F | 2.9 ± 0.2 | 0.0038 ± 0.000 | 0.0013 | 0.002 | 0.88 ± 0.00 | 0.62 ± 0.00 | 0.71 | 1.2 |
| N462Q | 0.88 ± 0.00 | 0.62 ± 0.00 | 0.71 | 1.2 | ND | ND | ND | ND |
lous dispersion method using the mercury-derivative crystal data. Native and Ara, X₃, and X₆ complex structures were successively determined. The structure refinement statistics are summarized in Table 3. Recombinant ScAraf62A comprised a single polypeptide chain of 438 amino acids, Ala38–Arg475, in which the N-terminal residues Met1–Ala37 constituted the signal peptide. The 136 N-terminal residues of Ala38–Asp173 comprising the linker and CBM13 domain were not identified because of a lack in the electron density. Therefore, only the structure of the C-terminal GH62 catalytic domain was determined. The final model included one ScAraf62A catalytic domain in the asymmetric unit, accompanied by one cation located at the center of the domain that was set as a calcium ion in this model, one chloride ion, and one citrate molecule. ScAraf62A is a monomeric protein in the native state. One disulfide bond (Cys176–Cys444) and one cis-peptide (Phe340–Pro341) were in the protein structure. The ligand-free structure contained one Tris molecule; the Ara complex structure contained one Ara molecule, and the X₃ complex structure contained one X₃ molecule. The X₆ complex structure contained one X₆ molecule, of which five xylose moieties were observed in the electron density map.

The ScAraf62A catalytic domain was a five-bladed β-propeller containing five radially oriented anti-parallel β-sheets, designated as blades I–V in Fig. 2; each blade essentially included four β-strands. The domain structure was primarily a β-structure with six β₁₀ helices and no α-helices. A fold such as this was first reported for tachylocin (52) and is observed so far in three glycose hydrolose families as follows: GH32, GH43, and GH68 (19, 53, 54). Overall, the structures of the sugar complexes were nearly identical to the ligand-free structure with root mean square differences of 0.12–0.15 Å, implying little effect of ligand binding on the overall structure.

| Data                  | Native   | Mercury derivative (peak) | Ara complex | X₃ complex | X₆ complex |
|-----------------------|----------|---------------------------|-------------|------------|------------|
| PDB code              | 3WNY     | 3WNO                      | 3WN1        | 3WN2       |
| Space group           | P4,2,2   | P4,2,2                    | P4,2,2      | P4,2,2     |
| Unit-cell parameters  | a = b = 97.2, c = 102.8 Å | a = b = 97.3, c = 103.5 Å | a = b = 98.2, c = 103.7 Å | a = b = 97.7, c = 104.0 Å |
| Beam line             | PF-AR NE3A | PF-AR NE3A              | PF BL-5A   | PF BL-5A   |
| Wavelength            | 1.0031 Å | 1.0031 Å                  | 1.0000 Å   | 1.0000 Å   |
| Resolution            | 100.0 to 1.40 Å (1.43 to 1.40 Å) | 100.0 to 1.90 Å (1.97 to 1.90 Å) | 100.0 to 1.90 Å (1.97 to 1.90 Å) | 100.0 to 2.00 Å (2.07 to 2.00 Å) |
| Rprog                 | 0.061 (0.441) | 0.077 (0.414)            | 0.106 (0.423) | 0.155 (0.451) |
| Completeness          | 95.9% (99.8%) | 99.9% (100.0%)           | 99.9% (100.0%) | 99.9% (100.0%) |
| Multiplicity          | 5.3 (5.0) | 28.5 (27.3)               | 12.0 (11.3) | 17.3 (17.3) |
| Average Fl (Å)        | 23.1 (6.6) | 42.9 (13.6)              | 20.4 (7.2)  | 42.9 (13.8) |
| Unique reflections     | 92,684 (6233) | 39,648 (3489)           | 40,681 (3984) | 34,678 (3400) |
| Observed reflections   | 488,849 | 1,131,521                 | 487,017     | 599,796    |

Table 3: Data acquisition and structure refinement statistics of α-L-arabinofuranosidase from Streptomyces coelicolor (ScAraf62A)

Values in parentheses refer to the highest resolution shell.

Crystal Structure of ScAraf62A in Complex with L-Arabinofuranose—L-Arabinofuranose was found in the catalytic pocket located at the central depression of the five blades (Figs. 2 and 3). Bound l-arabinofuranose assumed a structure of an envelope 2E conformation, and its O1 atom in the C1 hydroxyl group was observed in a β-anomeric configuration. This was docked in the pocket with four hydroxyl oxygen atoms oriented toward the bottom of the pocket, which were recognized by hydrogen bonds with the protein residues. The O1 atom of bound l-arabinofuranose (Ara-O1) was within the hydrogen bond distances with Asp202-O1, Lys201-N1, and Ser426-O2 atoms. One xylose moiety was hydrated the Glu361-O2e, Glu379-Oe1, and Ser426-Oy atoms hydrated the Ara-O1 and Ara-O2 atoms. The Ara-O3 atom hydrogen bonded to Asp399-O2 and His427-N01 atoms. One water molecule that was coordinated to Glu361-O2e, Lys201-N1, and Tyr461-Oy atoms hydrated the Ara-O1 and Ara-O2 atoms. The Ara-O3 atom hydrogen bonded to Asp399-O2, Glu361-O2e, and Ser426-Oy atoms hydrated the Ara-O1 and Ara-O2 atoms. The Ara-O5 atom hydrogen bonded to Asp202-O2 and Lys201-N1 atoms. The Ara-O4 atom, a component of the furanosyl ring, was within the hydrogen bond distances with the Lys201-N1 atom. Overall, the bound Ara molecule in the catalytic pocket was recognized by 11 direct hydrogen bonds and one water-mediated interaction. The C5 atom was sequestered in the hydrophobic hollow that included Tyr274, Trp790, and Val251. The flat surface of Ara was held from the top by the side chain of Ile308 by hydrophobic interaction.
positions were designated as subsites +3NR, +2NR, +1NR, +1, and +2R (Fig. 4, A and D). One surface of the xylose at subsite +1 was embedded in the aromatic ring of Tyr461, which formed a stacking interaction, and another side faced the side chains of Asp326 and Phe360. The O2 and O3 atoms faced toward the bottom of the cleft. The O2 atom hydrogen bonded to Glu361-O

\[ \text{Fo} - \text{Fc} \]  

electron density map of bound Ara (contour level, 1σ). Sugar carbon atoms are numbered.

FIGURE 2. Structure of α-L-arabinofuranosidase from S. coelicolor (ScAraf62A) catalytic domain. A, stereoview of the ribbon model of the ScAraf62A-α-L-arabinofuranose complex structure. The bound sugar, catalytically important residues, and disulfide bridge are shown as stick models, and the bound sodium ion is shown as a purple sphere. B, topological diagram of ScAraf62A. 3_10-helices and β-strands are shown as shaded cylinders and filled arrows, respectively.

FIGURE 3. Stereo view of the α-L-arabinofuranosidase from S. coelicolor (ScAraf62A)-L-arabinose complex catalytic pocket. Yellow stick model, bound Ara; pale red, three catalytically important residues; broken lines, estimated hydrogen bonds; magenta, 2Fo – Fc electron density map of bound Ara (contour level, 1σ). Sugar carbon atoms are numbered.
Protein side chains around the sugar-binding sites were mostly conserved among these structures, although an apparent structural difference was observed for Ile308 (Fig. 4C). In the ligand-free form, the major conformation of the Ile308 side chain was observed with a $\chi_1$ value of 51° (green stick model in Fig. 4C), but it was approximately between 161° and 163° in the Ara or xylooligosaccharide complex structures. The entrance to the catalytic pocket was wider in the former structure; therefore, the closed conformation in the latter structure appeared to be induced by the binding of sugar ligands either in the catalytic pocket or the catalytic cleft.

**ScAraf62A Mutagenesis Studies**—To evaluate the substrate specificity mechanism, some amino acids in ScAraf62A that were possibly involved in enzyme catalysis and substrate binding were mutated, and these mutant enzymes were characterized (Table 2). Three possible catalytic residues, Asp, Asp, and Glu, which are conserved in GH43 enzymes, were also conserved in ScAraf62A (Asp202, Asp309, and Glu361; Fig. 3). It was predicted that Asp202 and Glu361 behave as general base and acid catalytic residues, respectively (29). No enzyme activities for the mutant Asp202 and Glu361 enzymes, such as D202N and E361Q, were detected when enzyme activities were tested using PNP-$\alpha$-L-Araf.

Regarding substrate binding, the indole group of Trp270 had a stacking interaction with xylose at +3NR and formed a part of the pocket wall at subsite $-1$. When Trp270 was replaced by Ala, Tyr, or Phe, the $K_m$ value for PNP-$\alpha$-L-Araf significantly increased (5–9-fold), and the $k_{cat}$ value for the substrate slightly decreased (0.6–0.3-fold), whereas the values for wheat arabinoxylan were not significantly affected (3–5-fold for $K_m$ and 0.9–1.7-fold for $k_{cat}$). Because Tyr361 formed a stacking interaction with xylose located at subsite +1, Y461A, Y461F, and Y461W mutants were tested. Although all these mutants completely or significantly lost their activity for PNP-$\alpha$-L-Araf and wheat arabinoxylan, Y461A and Y461F showed an apparent activity for wheat arabinoxylan ($k_{cat}/K_m$ of 0.2–0.8 for wild type). The Y461W mutant lost its activity for PNP-$\alpha$-L-Araf and wheat arabinoxylan, probably because the side chain of the amino acid was too large and destroyed the structure of the substrate-binding cleft in ScAraf62A.

In contrast, Asn462 mutations did not affect the activity for PNP-$\alpha$-L-Araf but apparently affected the activity for arabinoxylan. For the hydrogen bonding of the O3 atom of Asn462 with xylose-O3 at subsite +2NR, an N462Q mutant was constructed to investigate the effect of this interaction on enzyme activity. The $k_{cat}/K_m$ value of N462Q for PNP-$\alpha$-L-Araf was 0.71 mM$^{-1}$min$^{-1}$ (1.2 relative to that of wild type), whereas it was 1.8 mg$^{-1}$ml$^{-1}$min$^{-1}$ (0.7 relative to that of wild type) for arabinoxylan. More critical differences were observed when arabinoxylomannosaccharides such as A$_1$X$_2$ and A$_1$X$_3$ were used as substrates.
substrates for N462Q (Table 4). This mutant had the same level of catalytic efficiency for \( A_1X_2 \) with wild-type ScAraf62A, whereas the catalytic efficiency of the mutant for \( A_1X_3 \) was about one-third of that for the wild-type enzyme, suggesting that the hydrogen bonding between Asn462 and xylose at subsite +2NR had an important role in locating arabinosylxylan at the exact position in the substrate-binding cleft of ScAraf62A.

**DISCUSSION**

**Catalytic Mechanisms and Substrate Specificities of GH62 Enzymes**—The detailed reaction mechanisms of GH62 enzymes have not yet been determined. Here, we present the structure of a GH62 enzyme, \( \alpha L \)-Arabinofuranosidase from *Streptomyces coelicolor* (ScAraf62A) against the arabino-oligosaccharides \( A_1X_2 \) and \( A_1X_3 \).

In the ScAraf62A-Ara complex structure, the bound Ara molecule was found in the catalytic pocket situated in the catalytic domain, as it is often observed with exo-acting enzymes. This pocket provided space for only one Ara moiety, and a cleft specific for a xylan backbone was present outside the pocket. The docked Ara molecule assumed the \( E \)-configuration, and its O1 atom in the C1 hydroxyl group was in a \( \beta \)-anomeric conformation, although the amounts of Ara \( \alpha \)- and \( \beta \)-anomers were almost the same in the solution. Because the scissile bond was an \( \alpha \)-glycosidic bond, the bound Ara molecule having the \( \beta \)-anomeric conformation appeared to represent the product state of the inverted catalytic process. This inverting mechanism is deduced from the relative positions of the catalytic residues, the bound Ara molecule, and the bound water molecule, as described below.

In general, two acidic amino acids, either aspartate or glutamate, are employed by most inverting GHs to catalyze hydrolysis. One carboxylate protonates the scissile glycosidic oxygen atom and the other coordinates one water molecule, which acts as the nucleophile (55). The ScAraf62A-Ara complex structure showed that the C1 hydroxyl group of the bound \( \alpha L \)-arabinofuranose molecule assumed a \( \beta \)-anomeric conformation and that the distance between the Glu361-Oe1 and the Ara-C1 atoms was 3.8 Å. However, the distance between the Ara-O1 and Glu361-Oe1 atoms would be within the hydrogen bonding distance, assuming that the Ara-O1 atom was in the \( \alpha \)-anomeric conformation. Furthermore, Glu361-Oe1 atom hydrogen bonded to the Xyl-O3 atom of the bound \( X_3 \) at subsite +1 in the ScAraf62A-\( X_3 \) complex structure. \( \alpha L \)-Arabinofuranosidase often substitutes the 3-hydroxyl group of the xylan backbone; thus, the atom at the O3 position could be considered as the scissile bond position of the substrate. Therefore, Glu361 appeared to play the proton donor role for this enzyme. However, one catalytic water molecule was necessary to invert the \( \alpha \)-anomeric conformation to the \( \beta \)-configuration by the catalytic process. In the ligand-free or ScAraf62A-\( X_6 \) complex structure, one bound water molecule was observed in proximity to the C1 atom of the bound Ara molecule in the direction of the \( \beta \)-anomeric side. This water molecule was closely supported by Asp202-Oe1, Lys201-Ne', Glu351-Ne2, and Tyr461-Oe atoms, and its distance was close to the Ara-O1 atom of the ScAraf62A-Ara complex structure. Therefore, it appears that the nucleophile water caused the inversion of the hydrolyzed \( \alpha L \)-arabinofuranose conformation, and Asp202 appeared to be the catalytic general base.

Besides the catalytic residues, acidic residues in the catalytic site often play important roles during catalysis, and their mutations cause drastic decreases in the hydrolytic activity of several GH enzymes. In ScAraf62A, Asp309 was located at the bottom of the catalytic pocket, bound to the Ara molecule by its O2 and O3 atoms, and appeared to be important for transition state stabilization. Asp309 also appeared to modulate the pKa of Glu361 and to keep it in the correct orientation relative to the substrate, as reported for Asp285 in *Geobacillus stearothermophilus* xylosidase (22). Three acidic residues in GH62 enzymes are considered to be catalytically important residues, and our present structural study highlights the roles of these residues in catalysis.

\( \alpha L \)-Arabinofuranosidase often substitutes the 3-hydroxyl group of the xylan backbone, although the 2-hydroxyl group is occasionally substituted. In the ScAraf62A-\( X_6 \) complex structure, the O2 atom of the bound xylose moiety at subsite +1 faced toward the catalytic cleft. However, the distance to the Ara-C1 atom in the ScAraf62A-\( X_6 \) complex structure was rather long (4.9 Å) compared with that from the O3 atom (3.1 Å). Therefore, this enzyme did not appear to catalyze the \( \alpha 1,2 \)-glycosidic bond in this situation. However, it seems plausible that the xylan backbone could be reversely docked in the substrate cleft so that the atom at the O2 position moves to the O3 position in the ScAraf62A-\( X_6 \) complex structure, because of the high structural symmetry of \( D \)-xylopyranoside and a \( \beta 1,4 \)-xylan backbone. In fact, in our previous study, we showed that xyooligosaccharides can reversibly bind in the xylan-binding pocket of CBM13 linked to \( \beta 1,4 \)-xylanase using decorated xyooligosaccharides (56). Moreover, cleavage of arabinofuranosyl side chains linked to O2 and O3 of single-substituted xylose residues in arabinoxylan by GH62 arabinofuranosidase was previously reported (49).

The possibility that arabinan can bind to the substrate-binding cleft of ScAraf62A was analyzed by molecular modeling. As shown in Fig. 5, arabinan is unable to fit into the cleft because of structural issues. In contrast to a xylan chain having an elongated structure with a typical 3-fold screw helix, an arabinan chain is extended with a turn to nest Ara residues in a different orientation with xylan. When the orientations of O2 and O3 of Ara located at subsite +1 were superimposed on the xylose of subsite +1 in the X3 binding model of ScAraf62A, the \( \alpha 1,5 \)-linked arabinan backbone did not fit into the substrate-binding cleft. Thus, ScAraf62A could discriminate sugar structures at the cleft, thereby enabling the hydrolysis of only arabinoxylan. As described above, the substrate-binding cleft of ScAraf62A appeared to play an important role in the substrate specificity of this enzyme. Our mutagenesis studies were in agreement with the structural insights into the substrate recognition mecha-
significant reduction in the activity for PNP-\(\alpha\)-1-Araf and a lesser effect on the activity for arabinoxylan were observed when Trp\(^{270}\) and Tyr\(^{461}\) were mutated. For the Trp\(^{270}\) mutants, a mutation may have caused a loss of the stacking interaction with the xylose at +3NR and disruption of the pocket structure for Ara binding. Therefore, this mutation would drastically affect small substrates that use only subsites +1 and −1 than polysaccharides in which only one subsite is lost among five subsites. Similarly, the affinity of subsite +1 should have been lost in the Tyr\(^{461}\) mutant, which would result in the reduction of activity against small substrates but not against polysaccharides. Asn\(^{462}\) mutations break the hydrogen bond between Asn\(^{462}\) and xylose at subsite +2NR and affect the activity only for polysaccharides. Furthermore, a reduction in the \(k_{\text{cat}}/K_m\) value for arabinoxylan was observed with the N426Q mutant, despite that the \(k_{\text{cat}}/K_m\) value for PNP-\(\alpha\)-1-Araf was unchanged. The importance of this hydrogen bond was also confirmed using a different approach from the substrate side.

Wild-type ScAraf62A cleaved \(A_1X_3\) at a 5.4-fold faster rate than \(A_1X_1\) (Table 4). Although xylose residues docked into subsites +2R, +1, and +2NR when the enzyme hydrolyzed \(A_1X_3\), only +2R and +1 were used for \(A_1X_2\) hydrolysis. This difference in catalytic efficiency is caused by the affinity for subsite +2NR, which primarily includes hydrogen bonding between Asn\(^{462}\) and xylose. A more dramatic reduction in the catalytic efficiency of N426Q was observed for \(A_1X_3\) than that for \(A_1X_2\).

Comparisons with GH51 and GH54 Arabinofuranosidases—Beldman et al. (57) divided arabinofuranosidases into three types as follows: type A (cannot degrade polymers), type B (can degrade polymers), and type AXH (specifically degrades arabinoxylan). Types A and B correspond to GH51 and GH54, respectively, and type AXH activity has been observed with GH43 and GH62 family enzymes.

The structure of the GH51 \(\alpha\)-1-arabinofuranosidase AbfA from \(G.\) stearothermophilus was the first structure resolved for an arabinoferanase. AbfA is a homohexamer, and each AbfA monomeric subunit is organized into a \((\beta/\alpha)_7\)-TIM barrel catalytic domain at the N terminus and a 12-stranded \(\beta\)-sandwich with a jelly roll topology at the C terminus (58). GH51 enzymes have a substrate-binding pocket that is suitable for binding to a single arabinofuranose residue. The pocket topology is not amenable for discriminating the structures of aglycons; therefore, this enzyme has broad substrate specificity for arabinose-containing substrates. However, there have been no detailed studies related to its structure and substrate specificity. In contrast, GH54 arabinofuranosidase can degrade polysaccharides without discriminating between xylan and arabinan. The crystal structure of the arabinofuranosidase from \textit{Aspergillus kawachi} showed that GH54 comprises a catalytic domain with a \(\beta\)-sandwich fold and an Ara binding domain belonging to CBM42 (59). CBM42 binds to arabinosyl side chains in arabinoxylans, and this property explains why GH54 enzymes can possibly degrade polysaccharides, although the enzymes in this family possess similar pocket topology for substrate binding (60). An Ara soaking study elucidated a small catalytic pocket in the catalytic domain, although the location of subsite +1 could not be confirmed. A loose interaction at subsite +1 may enable the hydrolysis of both \(\alpha\)-1,2- and \(\alpha\)-1,3-arabinofuranosyl linkages observed in the arabinoside side chains of arabinoxylans. Thus, the substrate recognition mechanism of GH62 is distinguishable from that of GH51 and GH54 enzymes by having a substrate-binding cleft that can fit the xylan backbone along with the arabinofuranose-binding pocket.
in GH62 appear to possess additional structural variations and have a unique recognition property for arabinobioxyln.

Conclusions—During the submission of our manuscript, the first structures for GH62 enzymes were reported (61). In our study, although the N-terminal CBM13 was not observed because of a lack of electron density, we determined the crystal structure of the ScAraf62A catalytic domain. α-L-Arabinofuranose is bound in the catalytic pocket, whereas xylooligosaccharide is bound in the cleft lying over the catalytic pocket. When α-1,5-linked α-arabinooligosaccharides were soaked into a ScArf62A crystal, we did not observe a clear electron density in the cleft. This discrimination of substrates was also apparent from the data of our mutagenesis study. As shown for the N462 mutant (Table 4), xylan recognition at subsite +2NR is important for substrate specificity in this enzyme, and this enzyme exhibits high hydrolysis activity toward arabinobioxyln but lesser activity toward arabinan. These results clearly explain the substrate discrimination mechanisms of GH62 enzymes that specifically hydrolyze arabinobioxylns but not arabinans.

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FIGURE 6. Superimposition of α-L-arabinofuranosidase from S. coelicolor (ScAraf62A) and two GH43 AXH catalytic domain structures. A, overall ribbon model. ScAraf62A-xylahexose (X6) complex, brown and pink; B, subtilis arabinobioxyln α-1,3-arabinofuranohydrolase complexed with xylotetraose (PDB code 3C7G) (25), cyan and blue; H. insoles double substituted xylan α-1,3-arabinofuranosidase complexed with 33α-arabinofuranosylxylotriose (PDB code 3ZXK) (28), pale green and green. B, close-up view of the three catalytically important residues superimposed on the boundary L-arabinose (Ara) molecule in the ScAraf62A-Ara complex. C, close-up view of the catalytic cleft with bound xylooligosaccharides.

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