Crystal structure of the catalytic domain of a GH16 β-agarase from a deep-sea bacterium, Microbulbifer thermotolerans JAMB-A94

Emiko Takagi1,2, Yuji Hatada1,2,* Masatake Akita1, Yukari Ohta1, Gaku Yokoi3, Takatsugu Miyazaki3, Atsushi Nishikawa3 and Takashi Tonozuka3,*

1Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Yokosuka, Japan; 2Graduate School of Nanobioscience, Yokohama City University, Yokohama, Japan; 3Department of Applied Biological Science, Tokyo University of Agriculture and Technology, Tokyo, Japan

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A deep-sea bacterium, Microbulbifer thermotolerans JAMB-A94, has a β-agarase (MtAgaA) belonging to the glycoside hydrolase family (GH) 16. The optimal temperature of this bacterium for growth is 43–49 °C, and MtAgaA is stable at 60 °C, which is one of the most thermostable enzymes among GH16 β-agarases. Here, we determined the catalytic domain structure of MtAgaA. MtAgaA consists of a β-jelly roll fold, as observed in other GH16 enzymes. The structure of MtAgaA was most similar to two β-agarases from Zobellia galactanivorans, ZgAgaA, and ZgAgaB. Although the catalytic cleft structure of MtAgaA was similar to ZgAgaA and ZgAgaB, residues at site 4 of MtAgaA were not conserved between them. Also, an α-helix, designated as α4’, was uniquely located near the catalytic cleft of MtAgaA. A comparison of the structures of the three enzymes suggested that multiple factors, including increased numbers of arginine and proline residues, could contribute to the thermostability of MtAgaA.

Key words: β-agarase; Microbulbifer thermotolerans; glycoside hydrolase family 16(GH16); thermostability; crystal structure

Introduction

Agarose is a major component of the polysaccharide complex agar.1-3 Agarose consists of β-galactopyranose (Gal) and 3, 6-anhydro-β-galactose (ALGal), which are composed of a repeating disaccharide unit [→3-Gal-β-(1→4)-α-ALGal]. β-Agarase hydrolyzes β-1,4 linkages in agarose to produce neoagarooligosaccharides. The Gram-negative deep-sea bacterium Microbulbifer thermotolerans JAMB-A94 was isolated from the sediment of Suruga Bay, Japan at a depth of 2406 m as an agar-degrading strain, and the optimal temperature for growth is 43–49 °C.4 We found two β-agarases from M. thermotolerans JAMB-A94, and they were designated as MtAgaA and MtAgaO.5,6 In the CAZY classification system (http://www.cazy.org/),7 MtAgaA and MtAgaO belong to the glycoside hydrolase families (GH) 16 and 86, respectively.5,6

The majority of the agarases are classified into GH16.1-3 MtAgaA consists of 433 amino acid residues including a signal peptide sequence of 18 residues.5 A homology search of the amino acid sequence shows that mature MtAgaA is divided into two parts, a GH16 catalytic domain (residues 19–300) and a carbohydrate binding module (CBM) 6 domain (residues 301–433). Heterologous expression of MtAgaA in Bacillus subtilis was carried out, but probably due to proteolytic cleavage, the expressed protein was detected as the catalytic domain composed of amino acid residues from 19 to 300. Characterization of the enzymatic properties showed that MtAgaA is stable at 60 °C, indicating that this enzyme is one of the most thermostable GH16 β-agarases.5 MtAgaA is therefore useful for recovering DNA from agarose gel electrophoresis, and is now commercially available (http://www.nippongene.com/pages/products/extraction/Thermo_b_agar/index_english.html). The structure of MtAgaA could provide a clue into the mechanisms of thermostability in GH16 enzymes. Several structures of GH16 enzymes are currently available. In particular, two β-agarases from Zobellia galactanivorans, ZgAgaA (52% identity with MtAgaA), and ZgAgaB (58% identity with MtAgaA) have been extensively studied8-10 and they display a β-jelly roll topology. Here, we determined the crystal structure of the catalytic domain of MtAgaA and compared it with ZgAgaA and ZgAgaB.

*Corresponding authors. Email: hataday@jamstec.go.jp (Y. Hatada); tonozuka@cc.tuat.ac.jp (T. Tonozuka)
Abbreviations: ALGal, 3,6-anhydro-β-galactose; Gal, β-galactopyranose; GH, glycoside hydrolase family; MtAgaA, Microbulbifer thermotolerans JAMB-A94 β-agarase A; rmsd, root mean square deviation; SdAgaB, Saccharophagus degradans 2-40 β-agarase B; ZgAgaA, Zobellia galactanivorans β-agarase A; ZgAgaB, Zobellia galactanivorans β-agarase B.

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Materials and methods

Expression of the enzyme. We previously constructed the expression plasmid, pBAG1, for the production of MtAgA in Bacillus subtilis ISW1214.5 The MtAgA gene was cloned in a SalI-BamHI site in pBAG1. To produce the enzyme more efficiently, pBAG1 was digested with SalI and BamHI, and the resulting fragment was ligated into the SalI-BamHI site of a high-expression B. subtilis vector, pEXBS.11 The plasmid, designated as pEXAG, was introduced into B. subtilis ISW1214. Expression of the enzyme was carried out in the same manner as in pBAG1.5

Purification of the enzyme. The culture supernatant (100 mL) was collected by centrifugation at 12000 × g for 20 min. Solid ammonium sulfate was added to the final concentration of 80% saturation, stirred at 4 °C for 1 h, and the precipitate was collected by centrifugation at 8000 × g for 20 min. After the precipitate was dissolved in 4 mL of 10 mM tris–HCl (pH 7.5), the solution was applied to a HiPrep 16/10 Phenyl FF (high sub) column (1.6 cm × 10 cm, GE Healthcare, Chalfont St Giles, UK) equilibrated with 0.2 mM ammonium sulfate and 10 mM tris–HCl (pH 7.5). The enzyme was eluted with a decreasing linear gradient from 0.2 to 0 mM ammonium sulfate in 10 mM tris–HCl (pH 7.5) at a flow rate of 1 mL/min. The active fractions were collected and applied to a MonoQ 10/100 GL column (1 cm × 10 cm, GE Healthcare). The enzyme was eluted with a linear gradient in the range 0–0.2 mM sodium chloride in 10 mM tris–HCl (pH 7.5) at a flow rate of 0.5 mL/min. The active fractions were collected and applied to a HiTrap Heparin HP column (5 mL column volume, GE Healthcare) equilibrated with 0.1 mM sodium phosphate buffer (pH 7.5). The column was washed with the same sodium phosphate buffer at a flow rate of 1 mL/min, and the flow-through fractions were collected. The activity was measured as described previously.5 The purified protein was detected as a single band on the SDS-PAGE. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard.

Crystallization, data collection, and model building. The purified enzyme was concentrated to about 15 mg/mL in 10 mM tris–HCl (pH 7.5) using an Amicon Ultra-15 10,000 NMWL ultrafiltration device (Merck Millipore, Darmstadt, Germany). The enzyme was crystallized at 10 °C using the hanging drop vapor diffusion method, where 1 µL protein was mixed with the same volume of well solution containing 1 M trisodium citrate, 0.3 M ammonium nitrate, 3% ethanol, and 100 mM HEPES buffer (pH 7.5). The obtained crystal was transferred to a cryo-solution of 30% (w/v) glycerol in well solution and flash frozen in a stream of nitrogen gas at 100 K. Diffraction data were collected on the beamline AR-NW12A, at the Photon Factory (Tsukuba, Japan). Data were processed and scaled with the program HKL200013 (Table 1). The structure of MtAgA was solved by molecular replacement using the program MOLREP13 in the CCP4 suite,14 and

| Table 1. Data collection and refinement statistics. |
|-----------------------------------------------|
| **Data collection**                            |
| Beamline                                       | PF-AR NW12A          |
| Wavelength                                     | 1.0                  |
| Space group                                    | P212121              |
| **Cell dimensions**                            |
| a (Å)                                         | 49.6                 |
| b (Å)                                         | 69.4                 |
| c (Å)                                         | 90.4                 |
| Resolution range (Å)                          | 50–1.60 (1.66–1.60)* |
| **Unique reflections**                        | 300,161              |
| **Redundance**                                 | 41,972               |
| **Completeness (%)**                          | 100 (100)*           |
| <(I)/o(I)>                                     | 59.5 (18.3)*         |
| Rmerge                                         | 0.031 (0.124)*       |
| **Refinement**                                 |
| Rwork                                         | 0.152                |
| Rfree                                         | 0.164                |
| Root mean square deviation (msd)               |                      |
| Bond lengths (Å)                               | 0.009                |
| Bond angles (°)                                | 1.46                 |
| Ramachandran plot (Molprobity)                | 98.6                 |
| Favored (%)                                    | 1.4                  |
| Allowed (%)                                    | 0                    |
| Outliers (%)                                   | 0                    |
| **Number of atoms**                            |
| Protein                                       | 2259                 |
| Sodium ion                                     | 2                    |
| Glycerol                                      | 12                   |
| Water                                         | 260                  |
| **Average B (Å²)**                             |
| Protein                                       | 12.1                 |
| Sodium ion                                     | 12.6                 |
| Glycerol                                      | 28.1                 |
| Water                                         | 23.5                 |

*The values for the highest resolution shells are listed in parentheses.

ZgAgA (PDB ID 4ATF)10 was employed as a probe model. Automated model building was performed with the program ARP/wARP.15 Refinement was carried out using the program REFMAC16 in the CCP4 suite, and manual adjustment and rebuilding of the model were carried out using the program Coot.17 Models for the ligands were built using 2Fo–Fo and Fo–Fc electron density maps, and the data processing and refinement statistics are listed in Table 1. Figures were generated using PyMOL (http://www.pymol.org/). Statistical values were calculated using the program ACT from CCP4 and the program VMD.18 Coordinates and structure factors of MtAgA were deposited in the Protein Data Bank under the accession code 3WZ1.

Results and discussion

Overall structure of MtAgA
The crystal structure of the catalytic domain of MtAgA (hereafter simply referred to as MtAgA) was determined at 1.6 Å resolution. The crystal contained a single molecule in the asymmetric unit. In the Ramachandran plot, 98.6 and 1.4% of residues were in the favored and allowed regions, respectively, and no residues were identified as outliers, as determined by the MolProbity server (http://molprobity.biochem.duke.edu/).19 The 2Fo–Fc electron density contoured at 1σ shows continuous electron density for almost all the amino acid
residues from Ala20 to Asn296, and two glycerol molecules from the cryoprotectant solution were found on the protein surface. A ribbon representation of MtAgaA is shown in Fig. 1(A). The overall fold can be defined as a β-jelly roll motif, and the structure is roughly composed of two antiparallel β-sheets, similar to other GH16 enzymes.\textsuperscript{2,3} α-helices and β-strands were numbered α1–α4 and β1–β17, respectively, based on the numbering scheme for ZgAgaA and ZgAgaB (Fig. 2).\textsuperscript{9} Near the helix α4, MtAgaA had a short additional helix, designated as α4’ (Fig. 1(A)). The two β-sheets were composed of the strands β3–β4–β5–β17–β7–β13–β14–β15 (sheet A) and β2–β6–β16–β8–β9–β10–β11–β12 (sheet B). A concave region formed by sheet B was identified as the active site of GH16 enzymes.\textsuperscript{2,3}

A structural comparison using the Dali server (http://ekhidna.biocenter.helsinki.fi/dali_server/)\textsuperscript{20} indicated that MtAgaA was most homologous to ZgAgaB (PDB ID 1O4Y; Z score ~25; rmsd ~2.0 Å) and endo-β-1,3-glucanases (e.g. PDB ID 2VY0,\textsuperscript{23}) these two atoms are likely sodium ions because refinement of these atoms as calcium ions produced strong negative $F_o - F_c$ maps. The B-factors of sodium ions at site-1 and site-2 were 10.8 and 14.4 Å$^2$, both of which were similar to the average B-factor of protein atoms (12.1 Å$^2$) (Table 1). The two metal-binding sites adopted penta-coordinate geometry that is typically observed in the calcium-binding sites based on the MESPEUS database.\textsuperscript{24} Site-1 is widely conserved among GH16 enzymes, and a calcium ion is predominantly observed at this site.\textsuperscript{3} The residues in site-1 are conserved among GH16 enzymes, and a calcium ion is predominantly observed at this site.\textsuperscript{3} The residues in site-1 are conserved among MtAgaA, ZgAgaA, and ZgAgaB. However, the conformation of the conserved residue Asn48 of MtAgaA is different from the corresponding residue in ZgAgaA and ZgAgaB. The distance between the atom at site-1 and atom OD of Asn48 was 4.1 Å, indicating that there is little or no interaction between the two atoms (Fig. 1(C)). As a result, the site did not adopt the hexacoordinate geometry that is typically observed in the calcium-binding sites.\textsuperscript{25}

The presence of the two sodium ions may be artifacts because of the high concentration (1 m) of trisodium citrate used for the crystallization. It is likely that the primary function of site-1 is a calcium-binding site. Our previous study showed that the enzymatic activity is not affected by the presence of 0.1 m sodium chloride.\textsuperscript{5}

\textit{Ligand-binding model of MtAgaA}

Based on the results of the structural comparison with ZgAgaA and ZgAgaB complexed with the ligands that have been determined previously, their catalytic residues, and subsite structures have been studied in detail.\textsuperscript{9–10} GH16 enzymes are retaining glycoside hydrolases, and two Glu residues were determined to be catalytic. On the basis of homology with ZgAgaA and ZgAgaB, Glu147 and Glu152 of MtAgaA are expected to function as a nucleophile and an acid/base catalyst, respectively (Fig. 2).

The structures of ZgAgaB substrate complex (PDB ID 4ATF) and MtAgaA were superposed and the coordinates of the agarose chain were then placed in the MtAgaA structure. The resulting model indicated

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**Fig. 1.** Overall structure of MtAgaA.

Notes: (A) A ribbon model of MtAgaA. α-Helices (α1, α2, α3, α4’, and α4), sodium binding sites (site-1 and site-2), two glycerol molecules (orange), N-terminus, and C-terminus are shown. (B) Stereo view of the superposition of the Ca backbones of MtAgaA (red), ZgAgaA (green), and ZgAgaB (blue). The dimer interface of ZgAgaB is indicated with a black arrow. Helix α4’ of MtAgaA and the corresponding structures of ZgAgaA and ZgAgaB are shown with a red arrow. (C, D) Sodium-binding site-1 (C) and site-2 (D) in MtAgaA. The $2F_o - F_c$ electron density maps are shown at a 1.5σ contour level.
that five Trp residues, Trp72, Trp138, Trp191, and Trp261 appeared to be important for the ligand binding (Fig. 3(A)). Eight subsites, from −4 to +4, have been proposed for ZgAgaA and ZgAgaB. Residues involved in subsites −3 to +4 of MtAgaA were essentially identical to ZgAgaA and ZgAgaB (Table S1). Two residues of ZgAgaA, Tyr69 and Glu144, were not conserved, and the corresponding residues in MtAgaA were identified as Phe68 and Ser144 (Fig. 3(A)). In the structure of ZgAgaA, complexed with an agaro-oligosaccharide, atom OE2 of Glu144 formed a hydrogen bond with atom O2 of ALGal at subsite −4, and both atom OH of Tyr69 and atom OE1 of Glu144 interacted with atom O2 of Gal at subsite −3. This result suggests that the specificity at subsites −3 and −4 of MtAgaA may be different from ZgAgaA and ZgAgaB. In the plus subsites, a relatively long loop was located at the entrance of the catalytic cleft (indicated by a red arrow in Figs. 1(A) and 3(A)). ZgAgaB had the longest loop (residues 310–326, 17 residues) and it creates a deep cleft, while the corresponding loop in ZgAgaA was short (residues 256–262, 7 residues), and thus the cleft was shallow. The length of this region in MtAgaA was in between ZgAgaA and ZgAgaB (residues 259–268, 10 residues) and formed a short helix, α4′ (underlined in Fig. 2). ZgAgaA has been proposed to prefer solid-phase agarose, while ZgAgaB has a greater efficiency for cleaving neoagarohexaose into neoagarotetraose and neoagaroobiose. The hydrolysis of neoagarohexaose by MtAgaA is slow based on the pattern of thin-layer chromatography, and also kinetic parameters indicated that MtAgaA prefers agar to neoagarohexaose. The enzymatic properties of MtAgaA appear to be, therefore, similar to those of ZgAgaA rather than those of ZgAgaB at a glance, but it is unclear whether the loop length is related to the substrate specificity of these enzymes.

It has been reported that ZgAgaA has a second substrate-binding site, which is proposed to enable the enzyme to unwind the double-helical structure of agarose prior to catalytic cleavage, whereas ZgAgaB does not possess this second substrate-binding site. Two Trp residues, Trp87 and Trp277, in ZgAgaA make stacking interactions with the agarose chain, and the side chains of Asn89, Gln92, and Asp271 in ZgAgaA form hydrogen bonds with the agarose chain. In contrast, in ZgAgaB, the positions equivalent to Trp87, Asn89, Gln92, Asp271, and Trp277 in ZgAgaA are Tyr123, Ala125, Gln128, Ile335, and Gly341, respectively.
respectively. The equivalent residues in MtAgaA appeared to be Tyr86, Ser88, Lys91, Pro277, and Tyr283, respectively, indicating that none of them are identical to those of ZgAgaA (Fig. 3(B)). These findings suggest that MtAgaA has either a weak or no substrate-binding site on the molecular surface. Unlike ZgAgaA, MtAgaA has a CBM6 domain at the C-terminus.5) The CBM6 domain, CBM6-2, of Saccharophagus degradans 2–40 AgaB (SdAgaB) was shown to bind specifically to the non-reducing end of agarose chains.26) Alignment of CBM6 domains of MtAgaA and SdAgaB shows that amino acid residues involved in the ligand binding are fully conserved in MtAgaA (Fig. S1). It is likely that the C-terminal, CBM6 domain in MtAgaA, may perform a similar function to the second substrate-binding site in ZgAgaA.

It has been reported to be relatively unusual for agarases to have a CBM6 domain; however, SdAgaB carries two CBM6 domains in the C-terminus.5) A study of the agarase metabolism of S. degradans 2–40 showed that SdAgaB is a major secreted agarose depolymerase in S. degradans 2–40, and a GH86 enzyme, Aga86E, occupies an intermediated step in the degradation of agarose.27) Alignment of the primary structures of ZgAgaA and SdAgaB indicated that Trp87, Asn89, Gln92, Asp271, and Trp277 in ZgAgaA were not conserved in SdAgaB (Fig. 2). Also, the catalytic domain of SdAgaB is highly similar to MtAgaA (65% identity). These results suggest that the physiological role of MtAgaA in M. thermotolerans JAMB-A94 is similar to SdAgaB in S. degradans 2-40. M. thermotolerans JAMB-A94 also has a GH86 enzyme, MtAgaO, which is homologous to Aga86E of S. degradans 2-40 (60% identity). Although it is unclear whether M. thermotolerans JAMB-A94 lives near the hydrothermal vent area, the optimal temperature for growth, 43–49 °C, is higher than many other Microbulbifer species.4) Despite the difference of the growth conditions, the results here suggest that there is a similarity between the agarase-degrading system of M. thermotolerans JAMB-A94 and that of S. degradans 2-40 (formerly known as Microbulbifer degradans 2-40), isolated from the surface of decomposing saltwater cordgrass in a Chesapeake Bay salt marsh.21,28)
Thermostability

Although the protein thermostability is affected by many complicated factors,29,30 several structural components, such as hydrogen bonds, salt bridges, disulfide bonds, and Pro and Arg residues, have been shown to be related to the stability of proteins.31–37 MtAgaA is stable to 60 °C,5 while ZgAgaA and ZgAgaB are stable to 45 °C. Mutagenesis studies of ZgAgaA and ZgAgaB have indicated that replacement of Ser63→Lys in ZgAgaA, Glu99→Lys in ZgAgaB, and Thr307→Ile in ZgAgaB is effective for thermostabilization.38,39 The corresponding positions of these residues in MtAgaA/ZgAgaA/ZgAgaB are Glu62/Ser63/Glu99 and Met256/Cys253/Thr307. Since these corresponding residues lack Lys and Ile, the MtAgaA thermostabilization mechanism is likely different from the thermostabilized ZgAgaA and ZgAgaB mutants.

Tabulations of the structural features of MtAgaA, ZgAgaA (PDB ID 1O4Y), and ZgAgaB (PDB ID 1O4Z) are listed in Table 2. The positions of the N-terminus and the C-terminus of these structural coordinates are almost identical when the three structures are superposed (Fig. 1(B)), suggesting that comparison of these three structures is appropriate for elucidating their stabilities. In general, increased numbers of hydrogen bonds, salt bridges,31,32 and decreased length of surface loops13) confer favorable effects for protein thermostability. Table 2 shows that ZgAgaA has the shortest length (270 residues), whereas ZgAgaB possesses the most numerous hydrogen bonds (472 bonds) and 20 salt bridges. The length of the primary structure of MtAgaA (277 residues) is almost identical to ZgAgaA (Fig. 1(B) and Table 2), and the numbers of hydrogen bonds (472 bonds) and salt bridges (18 salt bridges) are similar to those of ZgAgaB. Therefore, MtAgaA has a combination of favorable features, relatively abundant hydrogen bonds, salt bridges, and compact protein structure, for the protein thermostability.

Table 2. Proline residues located at position i + 1 of β-turns in MtAgaA.

| Turn-type | MtAgaA | ZgAgaA | ZgAgaB |
|-----------|--------|--------|--------|
| I         | 41–44 HPLS | 41–44 QDNVa | 78–81 QEIS |
| II        | 99–102 KPGT | 99–102 PAGNα | 136–139 KPGS |
| II        | 158–161 RPGQ | 157–160 DRGGα | 195–198 YSESα |
| I         | 213–216 DPWH | 211–214 DPFT | 253–256 DPWH |
| I         | 236–239 DPNG | 233–236 DPNN | 276–279 DPKH |
| II        | 285–288 DPGNb | 285–288 DPGNb | 285–288 DPNHb |
| I         | 276–279 DPNR | 270–273 DDSK | 334–337 NIENα |

*These sequences do not form β-turn structures, although the residues are located at positions similar to the corresponding residues in MtAgaA.

αThis sequence is found in the dimer interface of ZgAgaB, and thus there is no corresponding sequence in MtAgaA and in ZgAgaB.

Table 3. Structural features potentially involved in protein stability.

| MtAgaA | ZgAgaA | ZgAgaB |
|--------|--------|--------|
| Hydrogen bonda | 472 | 451 | 478 |
| Salt bridgeb | 18 | 11 | 20 |
| Disulfide bond | 0 | 0 | 0 |
| Number of amino acid residues | Pro | 21 | 16 | 15 |
|          | Arg  | 14 | 10 | 12 |

*Counted using the program ACT from CCP4. Only hydrogen bonds formed directly between amino acid residues were counted.

αCounted using the program VMD.

shortest length (270 residues), whereas ZgAgaB possesses the most numerous hydrogen bonds (472 bonds) and 20 salt bridges. The length of the primary structure of MtAgaA (277 residues) is almost identical to ZgAgaA (Fig. 1(B) and Table 2), and the numbers of hydrogen bonds (472 bonds) and salt bridges (18 salt bridges) are similar to those of ZgAgaB. Therefore, MtAgaA has a combination of favorable features, relatively abundant hydrogen bonds, salt bridges, and compact protein structure, for the protein thermostability.

Table 2 also indicates that the numbers of Pro and Arg are most abundant in MtAgaA among the three enzymes. The proline residues in the surface loops34) and the arginine residues exposed on the protein surface37) have been reported to be associated with the protein thermostability. When compared to ZgAgaA and ZgAgaB, MtAgaA has four unique Arg (residues 59, 63, 158, and 279) and eight unique Pro (residues 42, 71, 82, 115, 159, 249, 277, and 294), all of which are exposed or half exposed on the protein surface as calculated using Areaimol from CCP4 (Table S2). These residues are uniformly distributed on the protein surface of MtAgaA (Fig. 4), and are likely to contribute to the thermostability of MtAgaA. Substitution of residues in β-turns with Pro has been well studied. β-turns are comprised of four residues numbered i, i + 1, i + 2, and i + 3, and the substitution of the i + 1 position of type I and type II β-turns with Pro increases the conformational stability of protein.35,36 A secondary structure analysis of MtAgaA, ZgAgaA, and ZgAgaB using the

Fig. 4. Arginine and proline residues uniquely identified in MtAgaA.

Notes: Colors: blue, Arg; red, Pro located at position i +1 of β-turns; yellow, all other Pro. The model of agarose chain is shown in magenta, and the subsite numbers are indicated.
Conclusions

The crystal structure of the catalytic domain of MtAgaA was determined in this study. The Cα backbone and the catalytic cleft of MtAgaA are similar to ZgAgaA and ZgAgaB. MtAgaA has a CBM6 domain at the C-terminus, and the residues involved in the second substrate-binding site in ZgAgaA were not conserved in the corresponding site in MtAgaA. The physiological role of MtAgaA was suggested to be similar to SdAgaB, which is the major secreted agarose depolymerase from S. degradans 2-40. Our results also suggest that the thermostability of MtAgaA is likely attributed to a combination of multiple factors including the relatively high numbers of hydrogen bonds and salt bridges, the relatively short length of the surface loops, and the increased number of Pro and Arg residues.

Supplemental material

The supplemental material for this paper is available at http://dx.doi.org/10.1080/09168451.2014.988680.

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