cDNA cloning and bacterial expression of an endo-β -1,4 -mannanase, AkMan, from *Aplysia kurodai*

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Abstract

Previously we isolated an endo-β-1,4-mannanase (EC 3.2.1.78), AkMan, from the digestive fluid of a common sea hare *Aplysia kurodai* and demonstrated that this enzyme had a broad pH optimum spanning 4.0 to 7.5 and an appreciably high heat stability in this pH range (Zahura et al., *Comp. Biochem. Physiol*., B157, 137-148 (2010)). In the present study, we cloned the cDNA encoding AkMan and constructed a bacterial expression system for this enzyme to enrich information about the primary structure and the characteristic properties of this enzyme. cDNA fragments encoding AkMan were amplified by PCR followed by 5'- and 3'-RACE PCRs from the *A. kurodai* hepatopancreas cDNA using degenerated primers designed on the basis of partial amino-acid sequences of AkMan. The cDNA including entire translational region of AkMan consisted of 1,392 bp and encoded 369 amino-acid residues. The N-terminal region of 17 residues of the deduced sequence except for the initiation Met was regarded as the signal peptide of AkMan and the mature enzyme region was considered to comprise 351 residues with a calculated molecular mass of 39961.96 Da. Comparison of the primary structure of AkMan with other β-1,4-mannanases indicated that AkMan belongs to the subfamily 10 of glycosyl-hydrolase-family-5 (GHF5). Phylogenetic analysis for the GHF5 β-1,4-mannanases indicated that AkMan together with other molluscan β-1,4-mannanases formed an independent clade of the subfamily 10 in the phylogenetic tree. The recombinant AkMan (recAkMan) was expressed with an *Escherichia coli* BL21(DE3)-pCold1 expression system as an N-terminal hexahistidine-tagged protein and purified by Ni-NTA affinity chromatography. The recAkMan showed the broad pH optimum in acidic pH range as did native AkMan; however, heat stability of recAkMan was considerably lower than that of native enzyme. This may indicate that the stability of AkMan is derived from an appropriate folding and/or some posttranslational modifications in *Aplysia* cells.

1. Introduction

β-Mannan, the second most abundant hemicellulose in nature, is classified into four types of mannopolysaccharides, i.e., linear mannan, galactomannan, glucomannan, and galactoglucomannan, on the bases of its branching structure and sugar composition (Moreira and Filho, 2008). Endo-β-1,4-mannanase (EC 3.2.1.78, term β-mannanase in the present paper) is the enzyme that hydrolyzes internal β-1-4-mannosyl linkages of β-mannan producing mannooligosaccharides such as mannotriose and mannobiose (McCleary, 1988; Stalbrand, 2003). This enzyme has been used for various bioprocesses in which the quality of products is improved *via* degradation of β-mannan, e.g., bleaching softwood pulps, declining viscosity of feeds and foods, and clarifying beverages (Dhawan and Kaur, 2007). A vast variety of bacteria, actinomycetes, yeasts and fungi produce β-mannanase and degrade β-mannan to assimilate it as carbon and energy sources (Talbot and Sygusch, 1990; Puchart et al., 2004). β-Mannanase is also produced by higher organisms like plants (Shimahara et al., 1975; Marraccini et al., 2001; Gong and Bewley, 2007; Yuan et al., 2007) and invertebrate animals (Yamaura and Matsumoto, 1993; Yamaura et al., 1996; Xu et al., 2002a; Ootsuka et al., 2006; Song et al., 2008; Zahura et al., 2010). Among the invertebrates, herbivorous mollusks appear to be the most prominent β-mannanase producers. These mollusks feed plant tissues and digest β-mannan with β-mannanases in their digestive fluid. To date, molluscan β-mannanases have been investigated with a terrestrial gastropod *Pomacea insularus* (Yamaura and Matsumoto, 1993); a freshwater gastropod *Biomphalaria glabrata* (Vergote et al., 2005); marine gastropods *Littorina brevicula* (Yamaura et al.,1996), *Haliotis discus hannai* (Ootsuka et al., 2006), and *Aplysia kurodai* (Zahura et al., 2010); and a marine bivalve *Mytilus edulis* (Xu et al., 2002a and b). Among the molluscan enzymes, complete primary structure was determined in MeMan5A from *M. edulis* (Xu et al., 2002a and b), HdMan from *H. discus hannai* (Ootsuka et al., 2006), and BgMan5A from *Biomphalaria glabrata* (Vergote et al., 2005). According to the hydrophobic cluster analysis for the primary structures, these molluscan enzymes have been classified under the glycosyl hydrolase family 5 (GHF5).

Previously, we isolated a β-mannanase, AkMan, from the digestive fluid of *A. kurodai* and found that this enzyme preferably degraded linear mannan from a green alga *Codium fragile* producing mannotriose and mannobiose as major end products (Zahura et al., 2010). Further, we found that AkMan was considerably acid stable, i.e., this enzyme showed a broad pH optimum spanning 4.0 to 7.5 and the activity was not declined in such pH range upon incubation at 40^{o}C for 20 min (Zahura et al., 2010). In contrast, abalone HdMan which we had previously isolated (Ootsuka et al., 2006) was not stable in an acidic pH range, i.e., HdMan had a narrow pH optimum at pH 7.5 and readily inactivated below pH 6. Such high acid stability of AkMan may be ascribable to the adaptation of this enzyme to the acidic pH condition of the digestive fluid. Actually, pH of digestive fluid of *A. kurodai* was usually pH 6 or less. The stability may also be related to higher habitat temperature of *A. kurodai*, e.g., the habitat temperature of *A. kurodai* in summer frequently increases above 30^{o}C. On the other hand, pH of the digestive fluid of *H. discus hannai* is usually in a range of 6–8 and habitat temperature is below 15^{o}C. These differences in the properties between AkMan and HdMan seem to be derived from the differences in their primary structures. The complete amino-acid sequence of HdMan was previously revealed by the cDNA method (Ootsuka et al., 2006); however, no entire amino-acid sequence data for AkMan is currently available.

Therefore, in the present study, we cloned the cDNA encoding AkMan to reveal the entire primary structure of this enzyme. Further, we constructed a bacterial expression system for AkMan to provide the bases for the future protein-engineering study on this enzyme.

2. Materials and methods

2.1. Materials

An *A. kurodai* (body length, ~13 cm) was collected from the coast of Hakodate, Hokkaido Prefecture of Japan in July 2008. After the animal was anaesthetized by cooling with ice-water for 20

104 min, hepatopancreas was dissected from the animal and frozen with liquid nitrogen. The frozen
105 hepatopancreas had been stored at -80^{o}C until the extraction of total RNA. TA PCR cloning kit (pTAC-
106 1) was purchased from Biodynamics (Tokyo, Japan). Oligotex-dT(30), cDNA synthesis kit, 5′- and 3′-
107 Full RACE kits, cold shock expression vector pCold1, restriction endonucleases, T4 DNA ligase,
108 agarose, *Escherichia coli* strains DH5α and BL21(DE3), were purchased from TaKaRa (Tokyo, Japan).
109 AmpliTaq Gold PCR Master Mix and BigDye-Terminator Cycle Sequencing kit were from Applied
110 Biosystems (Foster city, CA, USA). Ni-NTA resin was from Invitrogen (Carlsbad, CA, USA). Bacto
111 tryptone, Bacto yeast extract, and other reagents used were from Wako Pure Chemicals Industries Ltd.
112 (Osaka, Japan). Linear β-1,4-mannan was prepared from *C. fragile* according to the method of Love
113 and Percival (1964). Locust bean gum (galactomannan, mannose:galactose is ~4:1 (mol:mol)) was
114 kindly supplied by MRC POLYSACCHARIDE Co. Ltd. (Toyama, Japan).
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116 *2.2. cDNA cloning and nucleotide sequencing*
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118 Total RNA of *A. kurodai* was extracted from 1 g of hepatopancreas by the guanidinium
119 thiocyanate–phenol method (Chomczynski and Sacchi, 1987), and mRNA was selected from the total
120 RNA with Oligotex-dT(30) according to the manufacturer's protocol. Hepatopancreas cDNA was
121 synthesized from the mRNA with a cDNA synthesis kit (TaKaRa) and random primers. The PCR was
122 performed in 20 μL of reaction mixture containing 50 mM KCl, 15 mM Tris–HCl (pH 8.1), 0.2 mM
123 each of dATP, dTTP, dGTP, and dCTP, 2.5 mM $MgCl_2$, and 10 pmol primers, 20 ng hepatopancreas
124 cDNA, and 0.5 units AmpliTaq Gold DNA polymerase. A successive reaction at 94°C for 20 s, 55°C
125 for 20 s, and 72°C for 45 s was repeated for 30 cycles with Thermal Cycler Dice mini (TaKaRa).
126 Amplified cDNA fragments were separated by 1.2 % agarose-gel electrophoresis and cloned with
127 pTAC-1 vector and *E. coli* DH5α. The transformants were grown in 2×YT medium supplemented by
128 50 μg/mL ampicillin at 37^{o}C for 14 h. The plasmids extracted from the transformants were subjected to

the sequence analysis with BigDye-Terminator Cycle Sequencing kit and ABI 3130 xl Genetic Analyzer (Applied Biosystems). The 3'-RACE and 5'-RACE PCRs were performed with specific primers synthesized on the bases of the nucleotide sequences of the amplified cDNAs.

2.3. Phylogenetic analysis for GHF5 β-mannanases

Phylogenetic analysis for GHF5 β-mannanases from bacteria, fungi, plants and mollusks was carried out by ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-j.html). Phylogenetic tree was drawn by Tree View software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.4. Expression of recombinant AkMan

Recombinant AkMan (recAkMan) was produced as the mature enzyme form by using the *E. coli* BL21(DE3)-pCold1 expression system. Thus, the coding region for the mature AkMan was amplified from the cloned AkMan-cDNA by the PCR with a primer set, 5'-GCT**CATATG**AGACTGCACATAC-3' and 5'-GTATAATA**GGATCC**CCGGGTC-3', which include *Nde*I and *Bam*HI sites, respectively (indicated with bold letters). The amplified fragment, AkManEx-cDNA, was cloned with pTAC-1 and DH5α and the inserted DNA, i.e., AkManEx-cDNA, was excised from the plasmid by the digestion with *Nde*I and *Bam*HI. The AkManEx-cDNA was then ligated to pCold1 vector which had been digested with the same enzymes and introduced to *E. coli* BL21(DE3). The transformed *E. coli* was cultivated at 37^{o}C in a 250 mL 2 x YT medium and the expression of recAkMan was induced by lowering the cultivation temperature to 15^{o}C and adding 0.1 mM IPTG. By this procedure, an N-terminus hexahistidine-tagged recAkMan was produced. The cells expressing recAkMan were harvested by centrifugation (8000×g for 10 min) and suspended with 30 mL of a lysis buffer containing 50 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 10 mM imidazole, 1% Triton X-100,

and 0.01 mg/mL lysozyme. The suspension was sonicated for 30 sec 5 times at 20 kHz using VP-050 Homogenizer (TAITEC, Tokyo, Japan) with 1-min cooling intervals. The cell lysate was then centrifuged at 10,000×g for 10 min and the supernatant was mixed with 0.2 mL of Ni-NTA resin (Invitrogen) preequilibrated with 50 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 10 mM imidazole, and 1% Triton X-100 and left at 4 ^{o}C for 1 h with occasionally mixing. Then, the resin was set in the small column (0.5 cm x 2.5 cm) and washed with 50 mM sodium phosphate (pH 7.0) and 40 mM imidazole. The recAkMan adsorbed was eluted with 0.5 M NaCl containing 150 mM imidazole buffer (pH 7.0) and collected as 0.5-mL fractions. The fractions showing β-mannanase activity were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0).

2.5. SDS-PAGE

SDS-PAGE was carried out with 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gel according to the method of Porzio and Pearson (1977). After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol-10% (v/v) acetic acid, and the background of the gel was destained with 5% (v/v) methanol–7% (v/v) acetic acid. Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA), was used as a molecular mass marker.

2.6. Assay for β-mannanase activity

Standard assay for β-mannanase activity was carried out in a medium containing 0.5% (w/v) locust bean gum, 10 mM sodium phosphate buffer (pH 6.0), and an appropriate amount of enzyme (usually 0.2~0.5 units/mL) at 30^{o}C. The reducing sugar liberated by the hydrolysis of locust bean gum was determined by the method of Park and Johnson (1949). One unit of β-1,4-mannanase was defined as the amount of enzyme that liberates reducing sugars equivalent to 1.0 μmol of D-mannose per

minute at the standard condition. Temperature dependence of the enzyme was determined at 15–60^{o}C in the standard assay medium, while thermal stability was assessed by measuring the activity remaining after the incubation at 10–60^{o}C for 20 min. pH dependence of the enzyme was determined at 30^{o}C in reaction media adjusted to pH 4.0–6.0 with 10 mM sodium acetate, pH 6.0–8.5 with 10 mM sodium phosphate, and pH 8.5–11 with 10 mM glycine–NaOH. pH stability was assessed by measuring the activity remaining after the incubation at 40^{o}C for 20 min in 50 mM sodium phosphate buffer (pH 4.0 – 10.0). The average values of triplicate measurements were used as each activity value.

2.7. Thin-layer chromatography

Degradation products of locust bean gum (galactomannan) and mannooligosaccharides (disaccharide–hexasaccharide, M2–M6) produced by β-mannanase were analyzed by thin-layer chromatography (TLC). A substrate solution (0.5 mL) containing 0.5% (w/v) locust bean gum or mannooligosaccharides and 10 mM sodium phosphate (pH 7.0) was mixed with 25 μL (0.125 unit) of β-mannanase and allowed to react at 30^{o}C. At appropriate time intervals, 10 μL of the reaction mixture was withdrawn and heated at 100^{o}C for 2 min to terminate the reaction. The reaction mixtures (each 2 μL) were then spotted on the TLC-60 plate (Merck, Darmstadt, Germany) and developed with 1-butanol–acetic acid–water (2:1:1, v:v:v). The sugars separated on the plate were detected with 10% (v/v) sulfuric acid in ethanol.

2.8. Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard protein.

3. Results

3.1. Nucleotide sequence of AkMan-cDNA

The cDNA encoding the N-terminal region of AkMan (AkMan-cDNA-1, Fig. 1) was amplified by the degenerated forward and reverse primers, AkManFw and AkManRv, which were synthesized on the basis of the N-terminal and an internal amino-acid sequences of AkMan (Zahura et al., 2010), respectively (Table 1). AkMan-cDNA-1 consisted of 621 bp encoding the amino-acid sequence of 207 residues that shows 57% identity with the sequence from 41st to 248th residues of the abalone β-mannanase HdMan (Ootsuka et al., 2006). The 3'-RACE and 5'-RACE PCRs were performed with specific primers, AkMan(3)Fw, AkMan(5)Fw and AkMan(5)Rv, designed on the basis of the nucleotide sequence of AkMan-cDNA-1 (Table 1 and Fig. 1), and the resulted cDNAs, i.e., 3RACE-cDNA (678 bp) and 5RACE-cDNA (333 bp), respectively, were subjected to the sequence analysis (Fig. 1). By overlapping the nucleotide sequences of 5RACE-cDNA, AkMan-cDNA-1, and 3RACE-cDNA, in this order, the nucleotide sequence of total 1,392 bp was determined (Figs. 1 and 2). The reliability of this sequence was confirmed with AkManFull-cDNA (1,259bp) that was newly amplified with the specific primer-pair AkManFull5F and AkManFull3Rv (Table 1 and Fig. 1). The translational initiation codon ATG was seen in nucleotide positions from 82 to 84 and termination codon TGA was seen in nucleotide positions from 1,189 to 1,191 (Fig 2). In the 3'-terminal region, a putative polyadenylation signal sequence, AATAAA and a poly (A+) tail were found. Accordingly, the sequence of 369 amino-acid residues was deduced from the translational region of 1,110 bp. Since the N-terminal region of 17 residues except for the initiation Met was predicted as the signal peptide of AkMan by the Signal P 3.0 software (http://www.cbs.dtu.dk/services/SignalP/) and the N-terminus of the native AkMan begins from the 19th Arg of the deduced amino-acid sequence (Fig. 2), the mature AkMan was considered to consist of 351 residues with the calculated molecular mass of 39961.96 Da. The internal amino-acid

sequences of AkMan (L-1~L-4), which we previously determined with lysylendopeptidyl fragments (Zahura et al., 2010), were seen in the deduced sequence (Fig 2). Thus, it was confirmed that the cDNAs cloned in the present study were of AkMan. The nucleotide and deduced amino-acid sequences for AkMan-cDNA are available from DNA Data Bank of Japan with the accession number AB571101.

3.2. Comparison of primary structures of molluscan β-mannanases

The deduced amino-acid sequence of AkMan was aligned with other molluscan β-mannanases, i.e., HdMan (Ootsuka et al., 2006; DDBJ accession number, AB222081) from abalone *H. discuss hannai*, MeMan5A (Xu et al., 2002b; DDBJ accession number, AJ271365) from blue mussel *M. edulis* and BgMan5A (Vergote et al., 2005; DDBJ accession number, AY678121) from freshwater snail *B. glabrata*, by the ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-j.html) (Fig 3). AkMan showed 53.4% sequence identity with HdMan, 47.1% with MeMan5A, and 36.9% with BgMan5A. The residues which have been considered to participate in the catalytic action of GHF5 enzymes were conserved in AkMan as Glu162 (putative catalytic acid/base) and Glu293 (putative catalytic nucleophile). Conservative 5 amino-acid residues in the active site of GHF5 enzymes were also conserved in AkMan as Gly20, Arg60, His262, Tyr264 and Trp322. Accordingly, AkMan was confirmed to be a member of GHF5 enzymes like other molluscan β-mannanases. GHF5 involves a large number of β-mannanses from a variety of prokaryotic and eukaryotic sources. On the basis of the sequence identity, eukaryotic β-mannanses were further classified into subfamily 7 whereas prokaryotic β-mannanses were classified into subfamily 8 (Hilge et al., 1998). On the other hand, molluscan β-mannanses were classified into subfamily 10 since they showed sequence identity less than 15% with the enzymes from subfamilies 7 and 8 (Larsson et al., 2006). Thus, AkMan can be classified to subfamily 10 of GHF5 like other molluscan enzymes.

A phylogenetic tree for β-mannanases of GHF5 from bacteria, fungi, plant and mollusks was made by using the ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-j.html) and the Tree View software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Fig. 4). AkMan together with other molluscan β-mannanases formed an independent clade distinct from those of β-mannanases from fungi, plants, and the bacteria (Fig 4). These clades are expectedly consistent with the division of subfamilies 7, 8 and 10 of GHF5 β-mannanases.

3.3. Purification of recAkMan

recAkMan was expressed as a hexahistidine-tagged protein with the pCold1 expression system and purified by Ni-NTA affinity chromatography. SDS-PAGE for the protein samples in various purification steps are shown in Fig. 5. The purified recAkMan showed a single protein band with approximately 40,000 Da which was similar to the calculated molecular mass of recAkMan (39961.96 Da) and that of native enzyme (40 kDa, Zahura et al., 2010). Although more than 90% of the expressed protein was produced in the insoluble fraction, approximately 0.04 mg of recAkMan showing the specific activity 15.8 U/mg was purified by the affinity chromatography from the soluble fraction of the cell lysate obtained from 250 mL culture (Table 2).

3.4. Biochemical properties of recAkMan

Optimum pH of recAkMan was similar to that of native AkMan, i.e., recAkMan showed a broad pH optimum spanning 4.0 to 7.5 as did native AkMan (Fig 6A). Whereas, recAkMan showed an optimal temperature at 45^{o}C which is 10 degrees lower than that of native AkMan (Fig 6B). The thermal stability of recAkMan was also significantly lower than that of native AkMan, i.e., temperature that caused 50% decrease in the activity of recAkMan during 20-min incubation was 38^{o}C while that for native AkMan was 52^{o}C (Fig 6C). In addition, stability of recAkMan in alkaline pH range was also

277 lower than that of native one, i.e., the activity of native AkMan was practically unchanged during
278 incubation at 40^{o}C in pH 4-8 while the activity of recAkMan decreased above pH 6 (Fig 6D). These
279 results indicated that heat stability of recAkMan is considerably lower than that of native AkMan
280 although the pH dependence is comparable between recombinant and native enzymes.

281 To assess the substrate preference of recAkMan, linear β-mannan from *C. fragile*, locust bean gum
282 (galactomannan) and mannooligosaccharides (M2–M6) were degraded by recAkMan and the
283 degradation products were analyzed by TLC. As shown in Fig. 7A and B, recAkMan degraded linear β-
284 mannan producing mainly trisaccharide, disaccharide and tetrasaccharide. On the other hand,
285 recAkMan degraded locust bean gum producing trisaccharide and disaccharide along with a series of
286 intermediary oligosaccharides larger than trisaccharide. These degradation products were practically
287 the same as those produced by native AkMan (Zahura et al., 2010). When mannooligosaccharide were
288 used as substrates, only the oligosaccharides larger than tetrasaccharide were degraded (Fig. 7C).
289 Namely, mannopentaose and mannohexaose were degraded by recAkMan and mannotriose and
290 mannobiose were produced by the degradation. These results indicate that recAkMan recognizes the
291 mannopenntaose–mannohexaose unit of β-mannan and cleaves the central β-1,4-mannosyl linkage of
292 the mannohexaose unit. The substrate preference of recAkMan was also the same as that of native
293 AkMan (Zahura et al., 2010). Accordingly, we concluded that overall properties of recAkMan are
294 practically the same as those of native AkMan except for the thermal stability.
295
296 **4. Discussion**
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298 *Aplysia kurodai*, a herbivorous marine gastropod, is a good specimen for the studies on the
299 molluscan polysaccharide-degrading enzymes since it possesses various enzymes which can degrade
300 seaweeds' polysaccharides in the digestive fluid (Kumagai and Ojima, 2010; Rahman et al., 2010;
301 Zahura et al., 2010). Previously, we isolated an endo-β-1,4-mannanase, AkMan, from the digestive

fluid of *A. kurodai* and determined its basic properties (Zahura et al., 2010). The partial amino-acid sequences of AkMan suggested that this enzyme belongs to GHF5; however, the entire amino-acid sequence had not been determined yet. In the present study, we successfully cloned the cDNA encoding AkMan and could analyze its primary structure.

AkManFull-cDNA encoded 369 amino-acid residues which comprised a putative translational initiation Met, a putative signal peptide region of 17 residues, and a mature enzyme region of 351 residues. The molecular mass of the mature enzyme region calculated from the deduced sequence was 39961.96 Da. This was consistent with the molecular mass of native AkMan (approximately 40 kDa) estimated by SDS-PAGE (Zahura et al., 2010). The deduced amino-acid sequence of AkMan shared 37-53% identities with those of molluscan β-mannanases reported so far (Fig 3). By the comparison of the sequences among the molluscan enzymes, several highly conserved regions were found i.e., ^{17}FLSGGN^{22}, ^{55}GGNSMRLWIH^{64}, ^{132}KLQSYIDKAL^{141}, ^{160}MNEPEG^{165}, ^{186}SGSGWAG^{192}, ^{202}RFLNWQADAIK^{212}, ^{287}KPIVVGEF^{294} and ^{315}GYAGAWSW^{322} (Fig 3). The amino-acid identities of these regions among the molluscan enzymes were higher than 75%. The residues that have been demonstrated to serve as catalytic nucleophile and proton donor in GHF5 enzymes, i.e., Gly20, Arg60, His262, Tyr264 and Trp322 in AkMan, are all located in these conserved regions (Fig. 3). The central NEP sequence of ^{160}MNEPEG^{165} of AkMan was considered to correspond to the catalytic center of GHF5 enzymes (Py et al., 1991). GHF5 β-mannanases have been divided into subfamilies 7, 8 and 10. AkMan possessed the residues characteristic in subfamily 10, i.e., W190 and W225, which have been considered to locate at subsites +1 and +2, respectively (Larsson et al., 2006 and Song et al., 2008). Therefore, AkMan was regarded as a new member of subfamily 10 of GHF5. Phylogenetic analysis for GHF5 β-mannanases from various organisms demonstrated that they are divided into three major clades. Namely, β-mannanses from bacteria formed one clade (subfamily 8), fungal and plant β-mannanses formed another clade (subfamily 7), and molluscan enzymes formed a clade (subfamily 10) distinct from the former two (Fig. 4). Although AkMan and HdMan belong to the same subfamily, they showed

appreciably different biochemical properties with respect to the temperature and pH dependence as described in the introduction part (Zahura et al., 2010; Ootsuka et al., 2006). These differences were considered to be derived from the sequence dissimilarity between the two enzymes. Actually, AkMan and HdMan showed relatively low sequence identity, i.e., approximately 53%, and many amino-acid replacements were found between two enzymes (Fig. 3). These differences in the primary structure may relate to the differences in temperature and pH dependence between two enzymes; however, it was difficult to point out the specific amino-acid residues or regions responsible for the heat and acid stabilities of AkMan since too many amino-acid replacements were distributed almost equally over the sequence.

Recombinant AkMan could be produced with the cold inducible *E. coli* expression system although the yield was modest. The specific activity of the recombinant enzyme was 15.8 U/mg which is somewhat smaller than that of native AkMan, i.e., 27.3 U/mg (Zahura et al., 2010). This may be due to the difference in the temperature dependence between recAkMan and native AkMan (see Fig. 6A). Namely, in the standard assay temperature 30^{o}C, recAkMan exhibited 30% of the maximal activity whereas native AkMan exhibited 45% of the maximal activity. The recAkMan was similar to native AkMan with respect to the broad pH optimum, substrate preference, and degradation products of β-mannan and mannooligosaccharides. However, heat stability of recAkMan was considerably lower than that of native enzyme. The actual reason for the liability of recAkMan is currently obscure; however, we consider that the higher order structure of recAkMan produced in *E. coli* cell might be somewhat different from that of native AkMan produced in *Aplysia* cell. Otherwise, some posttranslational modifications that may have taken place in the *Aplysia* cell are lacking in *E. coli* cell.

The yield of recAkMan in the BL21(DE3)-pCold1 system was small in the purpose of practical applications of this enzyme. To increase the yield of recAkMan, use of different expression systems may be effective. Besides the BL21(DE3)-pCold1 system, we have tried an expression vector pET44a which is known to be useful for producing many eukaryote proteins as NusA-tagged

recombinants. However, NusA-AkMan was not produced as a soluble form. Since *E. coli* expression systems are generally known to give rise to difficulties upon expressing eukaryotic proteins (Georgiou and Valax, 1996), eukaryote expression systems like yeast and insect cell systems may be preferable for the expression of recAkMan. Previously, *Mytilus* β-mannanase, which was produced as inclusion bodies with the *E. coli* expression system (Xu et al., 2002b), was successfully expressed in a soluble form by *Pichia pastoris* expression system (Xu et al., 2002b). Now, we are trying to express AkMan in the *P. pastoris* expression system which may provide the high yield production for recAkMan and also improve heat stability of recAkMan.

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Legends to figures

Fig. 1. Schematic diagram of cDNA for AkMan. Closed and open boxes indicate coding and non-coding regions of AkMan-cDNA, respectively. The numbers in the top of the boxes indicate the nucleotide positions. Relative positions for AkMan-cDNA-1, 3RACE-cDNA, 5RACE-cDNA, and AkManFull-cDNA are shown by thin lines, while the primers used for PCRs are shown as thick lines.

Fig. 2. The nucleotide and deduced amino-acid sequences of AkMan cDNA. Residue numbers for both nucleotide and amino-acid are indicated to the right of each row. The translational initiation codon ATG, termination codon TGA, and a putative polyadenylation signal AATAAA are boxed. A putative signal peptide is indicated by a dotted underline. The amino acid sequences determined with intact AkMan (N-terminus) and peptide fragments L-1, L-2, L-3, and L-4 are indicated with lines under the amino-acid sequence. The positions of degenerated primers, AkManFw and AkManRv, are indicated with arrows below the amino acid sequence, whereas those of specific primers, AkManFull5Fw and AkManFull3Rv, are indicated with arrows above the nucleotide sequence.

Fig. 3. Comparison of amino acid sequences of AkMan and GHF5 molluscan mannanases. The amino-acid sequence of AkMan was aligned with those of HdMan (DDBJ accession number, AB222081), MeMan5A (DDBJ accession number, AJ271365) and BgMan5A (DDBJ accession number, AY678121). Identical, highly conservative, and conservative residues among sequences are indicated by asterisk (*), colon (:), and dot (.), respectively. Regions showing relatively high similarity (higher than 75%) among the sequences are boxed. The residues participate in catalytic action and conserved in active sites of the GHF5 enzymes are marked as dark shade.

Fig. 4. Phylogenetic relationship for the GHF5 β-mannanases. The neighbor joining tree was constructed by using ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-j.html) and Tree View software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) with the sequences of β-mannanases from *Bispora* sp. MEY-1(DDBJ accession number EU919724), *Aspergillus fumigatus* (DDBJ accession number EU925594), *Aspergillus niger* (DDBJ accession number FJ268574), *Aspergillus aculeatus* (DDBJ accession number L35487), *Lactuca sativa* (DDBJ accession number AJ315978), *Glycine max* (DDBJ accession number DQ812101), *Coffea arabica* (DDBJ accession number AJ293305), *Daucus carota* (DDBJ accession number AF545503), *Solanum lycopersicum* (DDBJ accession number AY102168), *Agaricus bisporus* (DDBJ accession number Z50095), *Clostridium cellulolyticum* (DDBJ accession number CP001348), *Thermoanaerobacterium polysaccharolyticum* (DDBJ accession number U82255), *Cellvibrio japonicus* (DDBJ accession number AY187032), *Bacillus circulans* (DDBJ accession number AY913796), *Bacillus* sp. JAMB-602 (DDBJ accession number AB119999), *Vibrio* sp. MA-138 (DDBJ accession number D86329), *Caldicellulosiruptor saccharolyticus* (DDBJ accession number L01257), *Caldibacillus cellulovorans* (DDBJ accession number AF163837), *Biomphalaria glabrata*, BgMan5A (DDBJ accession number AY678121), *Mytilus edulis*, MeMan5A (DDBJ accession number AJ271365), *Haliotis discus hannai*, HdMan (DDBJ accession number AB222081) and *Aplysia kurodai*, AkMan (DDBJ accession number AB571101). Subfamilies 7, 8, and 10 are indicated with dotted circles.

Fig. 5. SDS-PAGE analysis for recombinant AkMan. *E. coli.* cells expressing recAkMan were sonicated with the lysis buffer and recombinant AkMan was purified from the supernatant of cell lysate by Ni-NTA affinity chromatography as described in the text. Mk, protein mass markers; Soni, sonicated sample; Sup, supernatant of cell lysate; Ppt, precipitates of cell lysate after centrifugation; Pass, passed through proteins from Ni-NTA column; Wash, proteins eluted by the wash buffer; R, purified recombinant AkMan.

Fig. 6. Effects of temperature and pH on enzymatic activities of recAkMan and native AkMan. (A) pH dependences of recAkMan (■, ●, ▲) and native AkMan (□, ○, △). Activity was measured at 30°C in reaction media adjusted to pH 4.0–6.0 with 10 mM sodium acetate (■, □), pH 6.0–8.5 with 10 mM sodium phosphate (●, ○), and pH 8.5–11 with 10 mM glycine–NaOH (▲, △). (B) Temperature dependence of native and recAkMan. Activities of recAkMan (●) and native AkMan (○) was assayed at 15–60°C in a reaction medium containing 0.5% locust bean gum and 10 mM sodium phosphate buffer (pH 6.0). (C) Thermal stabilities of recAkMan and native AkMan. recAkMan (●) and native AkMan (○) were incubated at 10–60°C for 20 min and the remaining activity was then assayed in 10 mM sodium phosphate (pH 6.0), 0.5% locust bean gum and 1 U/mL enzyme at 30°C. (D) pH stability of recAkMan and native AkMan. recAkMan (●) and native AkMan (○) were incubated at 40°C for 20 min in 50 mM sodium phosphate buffer adjusted to pH 4.0–10.0. Then, the remaining activity was measured at 30°C in 50 mM sodium phosphate buffer (pH 6.0) containing 0.5% locust bean gum.

Fig. 7. Thin-layer chromatography for the degradation products of β-mannans and mannooligosaccharides produced by recAkMan. Each substrate (0.5%) in 10 mM sodium phosphate buffer (pH 7.0) was degraded by recAkMan at 30^{o}C for 0–24 h. Substrates used were (A) β-1,4-mannan from *C. fragile*, (B) locust bean gum, (C) mannobiose to mannohexose (M2–M6). Mannooligosaccharides were degraded at 30°C for 24 h. The reaction products (each 2μL) were then spotted on the TLC-60 plate and developed with 1-butanol-acetic acid-water (2:1:1, v:v:v:).

529 **Table 1.** Primers used for amplification of AkMan cDNA.

	Primer names	DNA sequences
AkMan cDNA-1	AkManFw	5′ -AAYYTNCCNTGGATGWSNTA- 3′ (NLPWMSY*)
	AkManRv	5′ -YTTNGGRTTCCANACNCCCAT- 3′ (MGVWNPK*)
cDNA-3RACE	AkMan(3)Fw	5′ -AGCTCTGAAGAATTCAGGTGCC- 3′
	3Adapt	5′ -CTGATCTAGAGGTACCGGATCC- 3′
cDNA-5RACE	AkMan(5)Fw	5′ -ACAAGGCACCATGCTCGAC- 3′
	AkMan(5)Rv	5′ -ATTGCGCTGCCATTGACCG- 3′
Full-RACE	AkManFull5Fw	5′ -ACTACGACGCGGTCCTACTC- 3′
	AkManFull3Rv	5′ -CGGGACTCTATATGTCTTGCC- 3′

530 W, adenine or thymine; Y, cytosine or thymine; M, adenine or cytosine; R, adenine or
531 guanine; S, cytosine or guanine and N, adenine or guanine or cytosine or thymine.
532 * Zahura et al., 2010.
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Table 2. Purification of recAkMan by Ni-NTA affinity chromatography.

Preparation	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell lysate[a]	68.04	6.85	0.10	100	1
recAkMan	0.038	0.60	15.80	8.76	157.9

[a]Cell lysate was obtained from 250 mL culture.

569 Fig. 1

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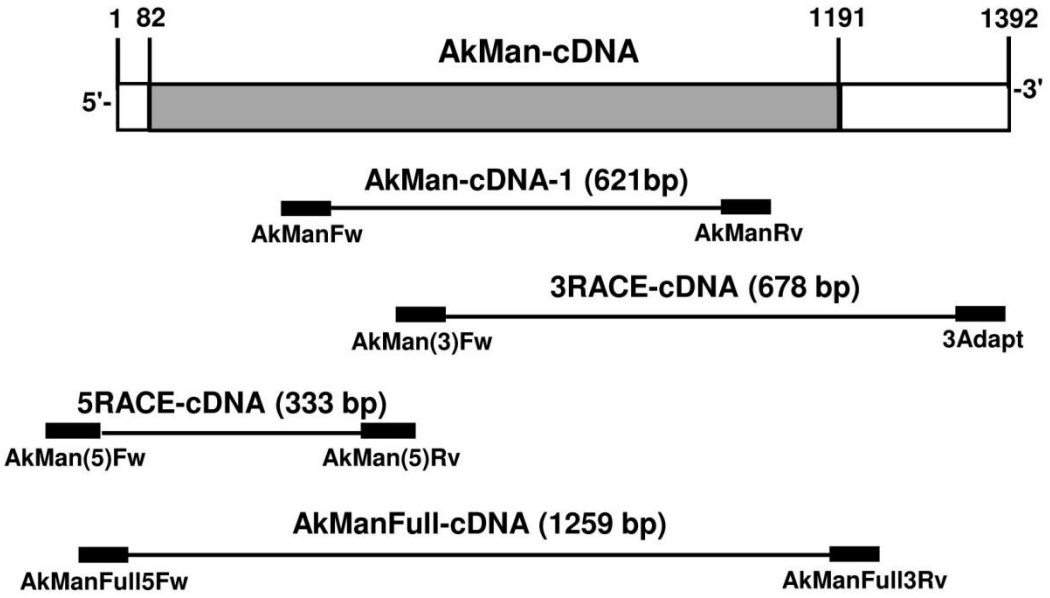

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588 Fig. 2

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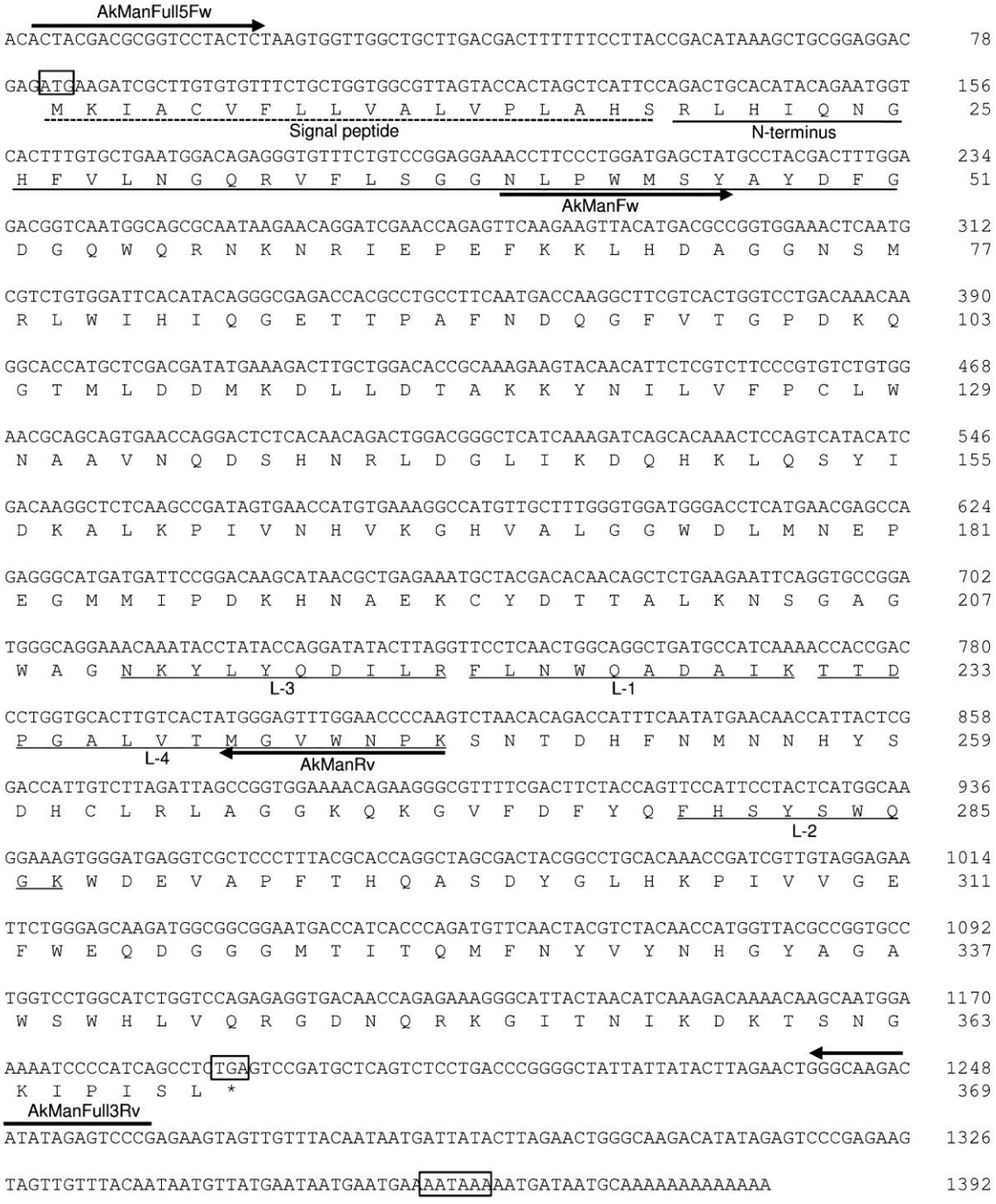

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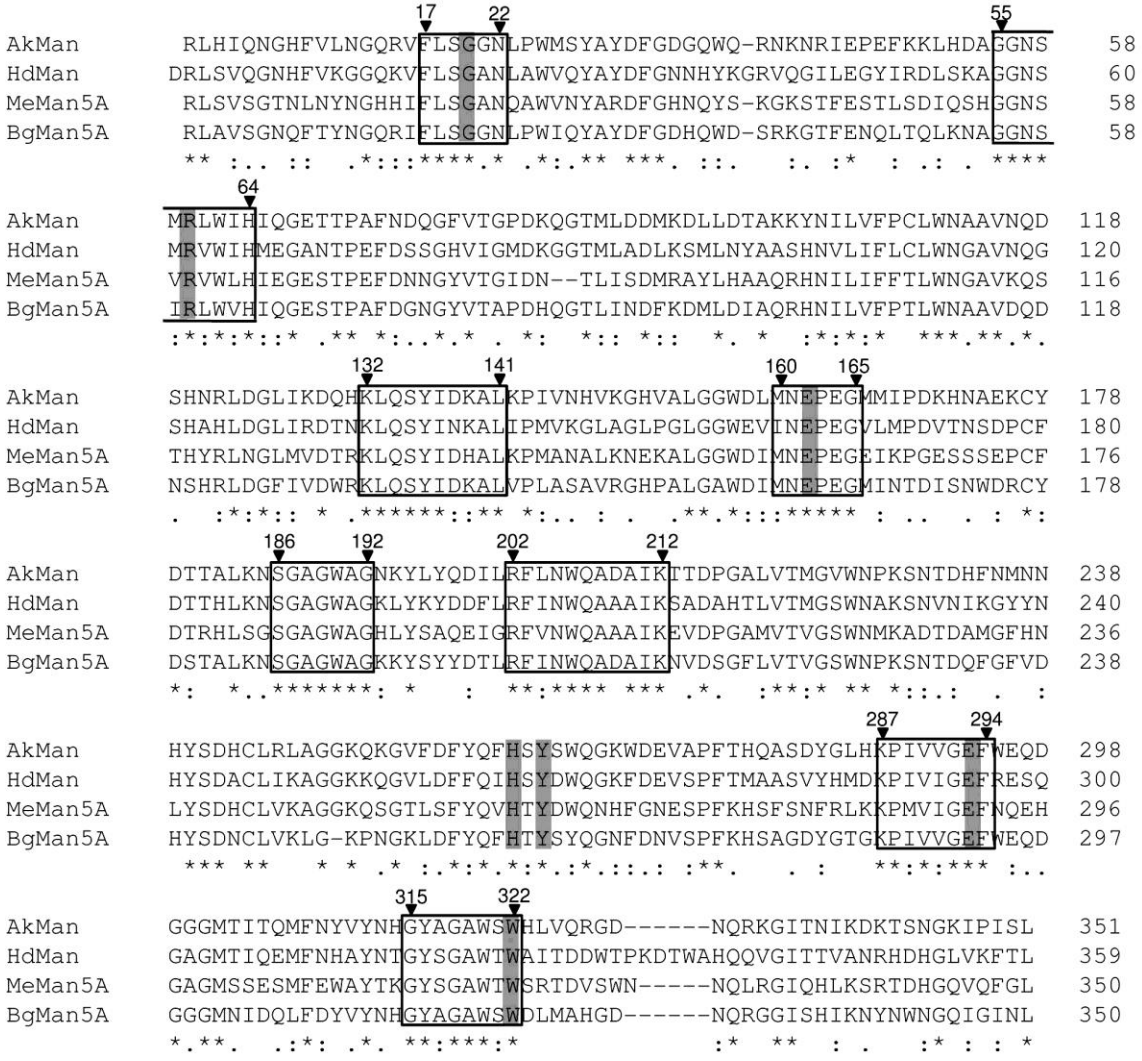
Fig. 3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AkMan RLHIQNGHFVLNGQRVFLSGGNLPWMSYAYDFGDGQWQ-RNKNRIEPEFKKLHDAGGNS 58
HdMan DRLSVQGNHFVKGGQKVFLSGANLAWVQYAYDFGNNHYKGRVQGILEGYIRDLSKAGGNS 60
MeMan5A RLSVSGTNLNYNGHHIFLSGANQAWVNYARDFGHNQYS-KGKSTFESTLSDIQSHGGNS 58
BgMan5A RLAVSGNQFTYNGQRIFLSGGNLPWIQYAYDFGDHQWD-SRKGTFENQLTQLKNAGGNS 58
AkMan MRLWIHIQGETTPAFNDQGFVTGPDKQGTMLDDMKDLLDTAKKYNILVFPCLWNAAVNQD 118
HdMan MRVWIHMEGANTPEFDSSGHVIGMDKGGTMLADLKSMLNYAASHNVLIFLCLWNGAVNQG 120
MeMan5A VRVWLHIEGESTPEFDNNGYVTGIDN--TLISDMRAYLHAAQRHNILIFFTLWNGAVKQS 116
BgMan5A IRLWVHIQGESTPAFDGNGYVTAPDHQGTLINDFKDMLDIAQRHNILVFPTLWNAAVDQD 118
AkMan SHNRLDGLIKDQHKLQSYIDKALKPIVNHVKGHVALGGWDLMNEPEGMMIPDKHNAEKCY 178
HdMan SHAHLDGLIRDTNKLQSYINKALIPMVKGLAGLPGLGGWEVINEPEGVLMPDVTNSDPCF 180
MeMan5A THYRLNGLMVDTRKLQSYIDHALKPMANALKNEKALGGWDIMNEPEGFIKPGESSSEPCF 176
BgMan5A NSHRLDGFIVDWRKLQSYIDKALVPLASAVRGHPALGAWDIMNEPEGMINTDISNWDRCY 178
AkMan DTTALKNSGAGWAGNKYLYQDILRFLNWQADAIKITDPGALVTMGVWNPKSNTDHFNMNN 238
HdMan DTTHLKNSGAGWAGKLYKYDDFLRFINWQAAAIKSADAHTLVTMGSWNAKSNVNIKGYYN 240
MeMan5A DTRHLSGSGAGWAGHLYSAQEIGRFVNWQAAAIKEVDPGAMVTVGSWNMKADTDAMGFHN 236
BgMan5A DSTALKNSGAGWAGKKYSYYDTLRFINWQADAIKNVDSGFLVTVGSWNPKSNTDQFGFVD 238
AkMan HYSDHCLRLAGGKQKGVFDFYQFHSYSWQGKWDEVAPFTHQASDYGLHKPIVVGEFWEQD 298
HdMan HYSDACLIKAGGKKQGVLDFFQIHSYDWQGKFDEVSPFTMAASVYHMDKPIVIGEFRESQ 300
MeMan5A LYSDHCLVKAGGKQSGTLSFYQVHTYDWQNHFGNESPFKHSFSNFRLKKPMVIGEFNQEH 296
BgMan5A HYSDNCLVKLG-KPNGKLDFYQFHTYSYQGNFDNVSPFKHSAGDYGTGKPIVVGEFWEQD 297
AkMan GGGMTITQMFNYVYNHGYAGAWSWHLVQRGD------NQRKGITNIKDKTSNGKIPISL 351
HdMan GAGMTIQEMFNHAYNTGYSGAWTWAITDDWTPKDTWAHQQVGITTVANRHDHGLVKFTL 359
MeMan5A GAGMSSESMFEWAYTKGYSGAWTWSRTDVSWN-----NQLRGIQHLKSRTDHGQVQFGL 350
BgMan5A GGGMNIDQLFDYVYNHGYAGAWSWDLMAHGD------NQRGGISHIKNYNWNGQIGINL 350

603 Fig. 4

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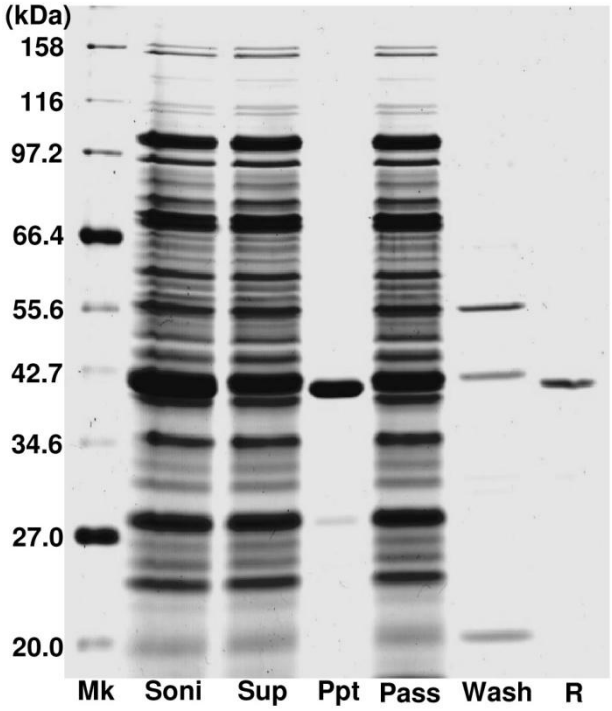
611 Fig. 5



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628 Fig. 6

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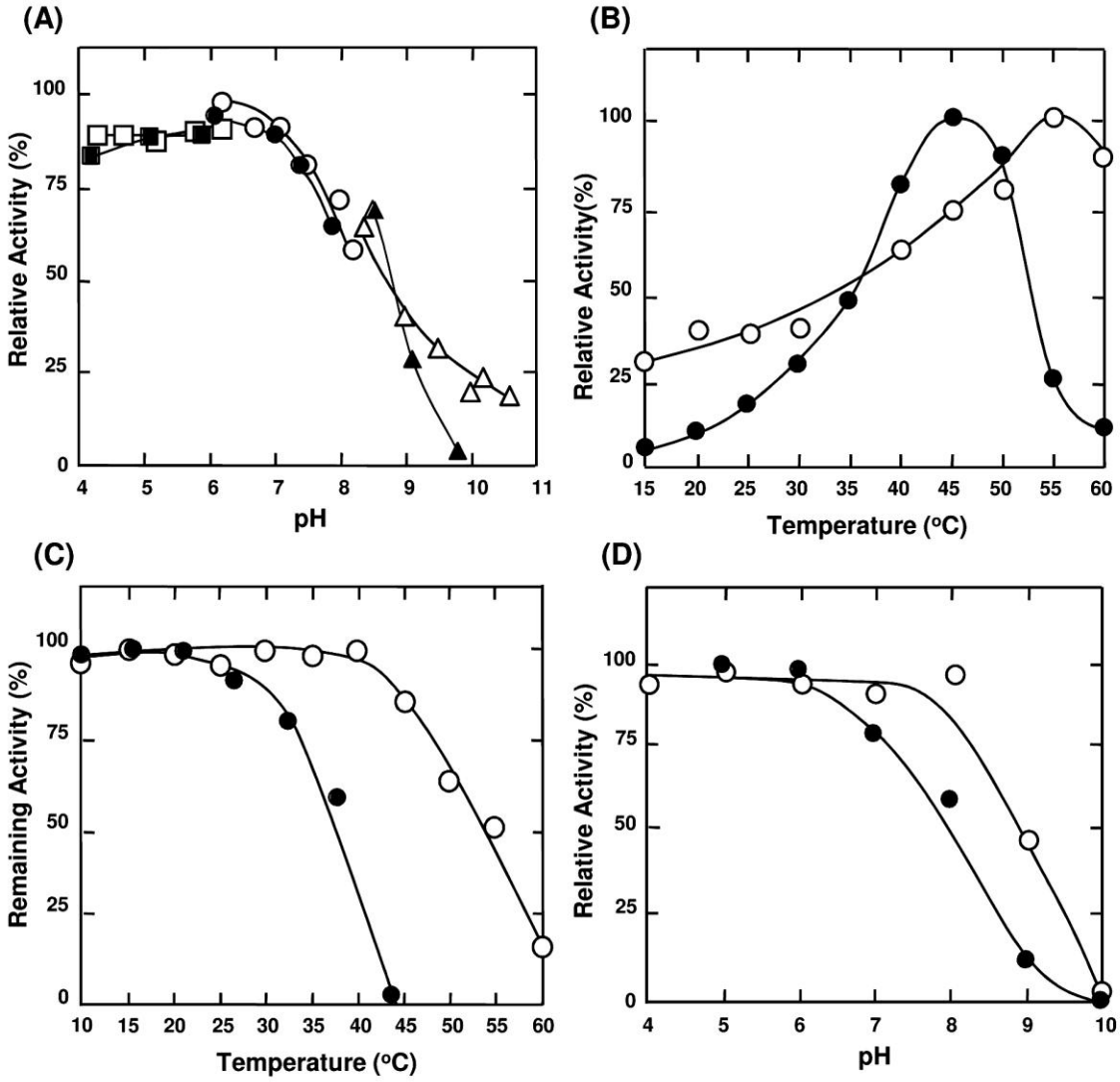

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639 Fig. 7

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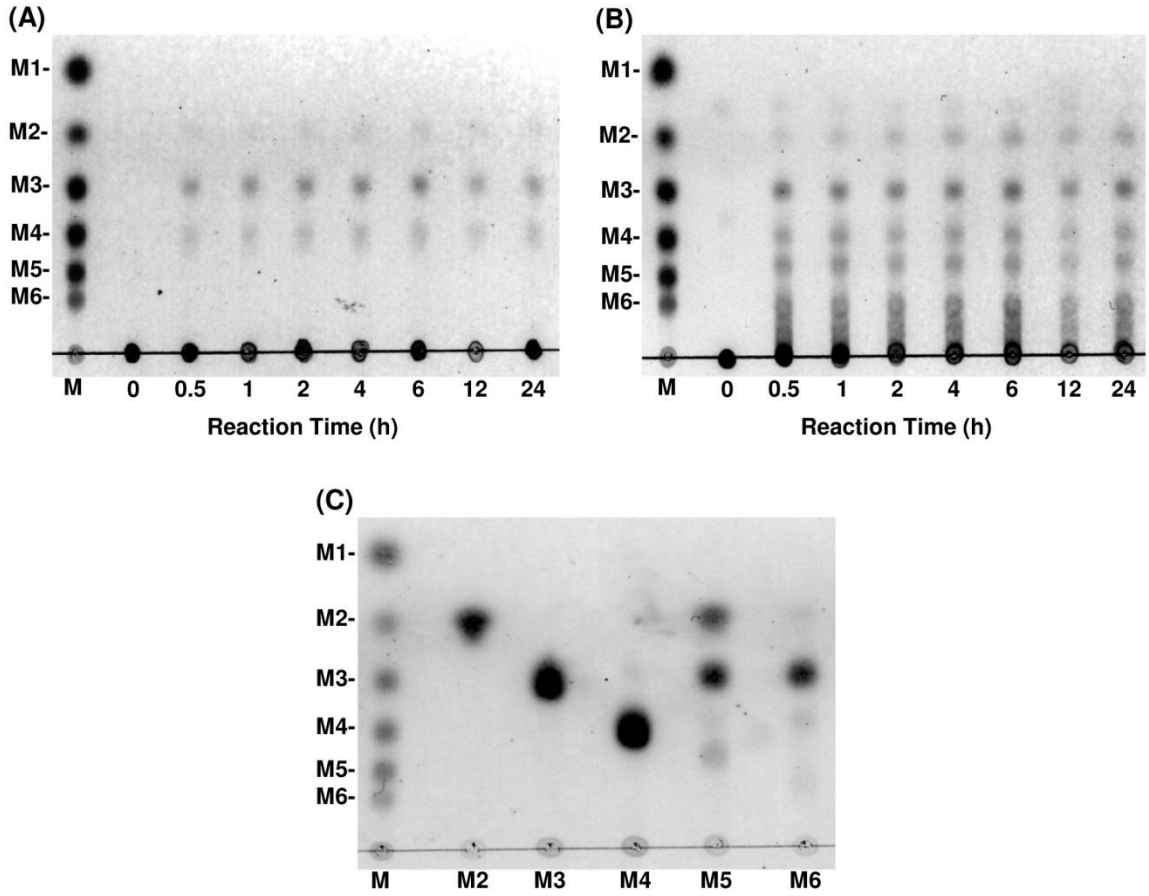

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