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**An endo- $\beta$ -1,4 mannanase, AkMan, from the common sea hare *Aplysia kurodai***

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## Abstract

A mannan-degrading enzyme was isolated from the digestive fluid of the common sea hare *Aplysia kurodai* by ammonium sulfate fractionation followed by conventional column chromatography. The purified enzyme, named AkMan in the present paper, showed a single band with an approximate molecular mass of 40,000 Da on SDS-PAGE and preferably degraded a linear  $\beta$ -1,4-mannan from green algae *Codium fragile* producing tri- and disaccharides. The optimal temperature of AkMan was 55 °C at pH 7.0 and temperature that caused 50% inactivation of AkMan during 20-min incubation was 52 °C. AkMan retained high activity at pH 4.0-7.5 and was not inactivated in such acidic pH range by the incubation at 40 °C for 20 min. AkMan could degrade glucomannan from konjak root and galactomannan (tara gum and guar gum) as well as the linear  $\beta$ -1,4-mannan, while not carboxymethyl cellulose, agarose, dextran and xylan. These results indicate that AkMan is a typical endo- $\beta$ -1,4-mannanase (EC 3.2.1.78) splitting internal  $\beta$ -1,4-mannosyl linkages of mannan. The N-terminal and internal amino-acid sequences of AkMan shared ~55% amino-acid identity to the corresponding sequences of an abalone  $\beta$ -1,4-mannanase, HdMan, which belongs to glycosyl-hydrolase-family 5 (GHF5). Thus, AkMan was also regarded as a member of GHF5  $\beta$ -1,4-mannanases.

## 1. Introduction

Mannan is a group of polysaccharides consisting of four different types of mannan, i.e., linear mannan, galactomannan, glucomannan, and galactoglucomannan (Moreira and Filho, 2008). Mannan exists as a structural polysaccharide in cell wall and/or intercellular matrices of both terrestrial and marine plants. Higher order structure of  $\alpha$ -mannan varies depending on its origin. For example, a linear mannan from a green seaweed *Codium fragile* is a homopolymer comprised of  $\beta$ -1,4-linked D-mannose residues while glucomannan from konjak root is a mixed polymer comprised of D-mannose and D-

glucose residues. In addition, the main chain of galactomannan such as locust bean gum and tara gum is modified by  $\beta$ -1,6-linked D-galactose branches (Moreira and Filho, 2008). Currently, mannan and its degradation products, i.e., mannoooligosaccharides, have been attracting attentions of researchers in the field of food and pharmaceutical industries because these poly- and oligosaccharides exhibit various beneficial effects on human health (Moreira and Filho, 2008; Dhawan and Kaur, 2007). For example, mannan alleviates intestinal disorders as a dietary fiber and mannoooligosaccharides improve intestinal microflora conditions as prebiotics. Consequently, a mannan-degrading enzyme,  $\beta$ -1,4-mannanase (EC 3.2.1.78), is also recognized as an important enzyme to produce the above bioactive mannoooligosaccharides. In addition, this enzyme has been demonstrated to be available in various industrial processes, e.g., clarifying of fruit juice, reducing viscosity of coffee extract, improving digestibility of poultry feeds, and bleaching pulp, where degradation of mannan improves the quality of products (Dhawan and Kaur, 2007).

$\beta$ -1,4-Mannanase has been isolated from bacteria (Araki, 1983 ; Akino et al., 1988; Talbot et al., 1990; Braithwaite et al., 1995; Nakajima and Matsuura, 1997; Li et al., 2000; Politz et al., 2000; Kansoh and Nagieb, 2004; Li et al., 2006), fungi (Johnson, 1990; Stalbrand et al., 1993; Kurakake et al., 2001; Puchart et al., 2004; Naganagouda et al., 2009), higher plants (Shimahara et al., 1975; Marraccini et al., 2001), and mollusks (Yamaura and Matsumoto, 1993; Yamaura et al., 1996; Xu et al., 2002a and b, and Ootsuka et al., 2006). Compared with bacterial and fungal enzymes, molluscan enzymes have not been well investigated, i.e., only four  $\beta$ -1,4-mannanases have been investigated in *Pomacea insularis* (Yamaura and Matsumoto, 1993), *Littorina brevicula* (Yamaura et al., 1996), *Mytilus edulis* (Xu et al., 2002a and b), and *Haliotis discus hannai* (Ootsuka et. al., 2006). An endo- $\beta$ -1,4-mannanase, HdMan, from the digestive fluid of the pacific abalone *H. discus hannai* showed a molecular mass of ~39 kDa on SDS-PAGE, and exhibited high hydrolytic activity on both galactomannan from locust bean gum and

glucomannan from konjak root. HdMan could degrade either  $\beta$ -1,4-mannan or  $\beta$ -1,4-mannooligosaccharides producing mannotriose and mannobiose (Ootsuka et. al., 2006). In addition, HdMan could disperse the fronds of a red alga *Porphyra yezoensis* into cell masses consisting of 10–20 cells that are available for cell engineering of this alga.

Seaweeds, the basis of the complex food web of marine environment, are eaten by the herbivorous marine gastropods such as abalone, periwinkle and sea hare. These gastropods possess various polysaccharide-degrading enzymes with different mode of actions and the gastropods degrade seaweeds' polysaccharides to obtain carbon and energy sources.  $\beta$ -1,4-Mannan exist as a structural polysaccharide in green and red seaweeds and the gastropods degrade this polysaccharide with  $\beta$ -1,4-mannanase. Thus,  $\beta$ -1,4-mannanase seems to be an important enzyme for the gastropods to assimilate seaweeds' fronds. To enrich the knowledge on molluscan  $\beta$ -1,4-mannanase, we consider that it is necessary to isolate the enzymes from as many mollusks as possible and investigate their general properties comparatively. Beside abalone, the common sea hare *Aplysia kurodai* seemed to be a good source for polysaccharide-degrading enzymes since the amount of digestive fluid from sea hare is significantly larger than that from abalone, e.g., ~10 ml from an average size sea hare (13 cm in body length) vs. ~1 ml from an abalone (5 x 8 cm in shell size). In addition, many animals are available in the shore of Hakodate, Hokkaido, in breeding season (June -July). Indeed, we recently succeeded to isolate  $\beta$ -1,3-glucanase, a laminarin-degrading enzyme, from the digestive fluid of *A. kurodai* (Kumagai and Ojima, 2010). Therefore, in the present study, we attempted to isolate and characterize  $\beta$ -1,4-mannanase from the digestive fluid of *A. kurodai*. Although there is a single report on the  $\beta$ -mannosidase from *A. fasciata* (Andreotti et al., 2005), no  $\beta$ -mannanase has been isolated from any *Aplysia* species. To our knowledge, this is the first report on the isolation and characterization of  $\beta$ -1,4-mannanase from sea hare.

## 2. Materials and methods

### 2.1. Materials

The common sea hare, *A. kurodai* (body length, ~13 cm) was collected in July 2008 in the shore of Hakodate, Hokkaido, Japan. Digestive fluid of the animal was obtained from their gastric lumen by squeezing the stomach after dissection. Approximately 107 ml of the digestive fluid was obtained from 10 animals. The digestive fluid was then dialyzed against 2 mM sodium phosphate buffer (pH 7.0) for 2 h and centrifuged at 10,000×g for 10 min to remove insoluble materials. The supernatant was used as a crude enzyme for the purification of  $\beta$ -1,4-mannanase. Locust bean gum (galactomannan with a molar ratio for mannose:galactose is ~4:1), tara gum (galactomannan, mannose:galactose is ~3:1) and guar gum (galactomannan, mannose:galactose is ~2:1), glucomannan (mannose:glucose is ~3:2) from konjak root were kindly supplied by MRC POLYSACCHARIDE Co. Ltd. (Toyama, Japan). Carboxymethyl cellulose (medium viscosity) was purchased from the ICN Biomedical, Inc. (Aurora, OH, USA), and  $\beta$ -1,4-mannooligosaccharides (mannobiose–mannohexaose, M2–M6) from Megazyme (Bray, Ireland). Dextran, agarose and xylan were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Linear  $\beta$ -1,4-mannan was prepared from *Codium fragile* according to the method of Love and Percival (1964). TOYOPEARL CM-650M and TOYOPEARL Phenyl-650M were from Toyo Soda Mfg. Co. (Tokyo, Japan), and Mono-S 5/50GL from GE Healthcare UK Ltd. (Little Chalfont, Bucking Hamshire, England). The other chemicals used were reagent grade from Wako Pure Chemical Industries Ltd.

### 2.2. Assay for $\beta$ -1,4-mannanase activity

Standard assay for  $\beta$ -1,4-mannanase activity was carried out in a medium containing 0.5% (w/v) locust bean gum, 10 mM sodium phosphate buffer (pH 7.0), and an appropriate amount of enzyme at 30 °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  $\beta$ -1,4-mannanase was defined as the amount of enzyme that liberated reducing sugars equivalent to 1.0  $\mu$ mol of D-mannose per minute. Temperature dependence of  $\beta$ -1,4-mannanase was determined at 15–60 °C in the standard assay medium, while thermal stability was assessed by measuring the remaining activity of the enzyme heated at 20–60 °C for 20 min in the standard assay medium. pH dependence of  $\beta$ -1,4-mannanase activity was determined at 30 °C in reaction media adjusted to pH 4.5–6.0 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 of AkMan was assessed by measuring the activity remaining after the incubation at 40 °C for 20 min in 50 mM sodium phosphate buffer adjusted to pH 4.0 – 10.0. The activity after the incubation was assayed at 30 °C in a medium containing 50 mM sodium phosphate buffer (pH 6.2) and 0.5% locust bean gum. Effects of metal ions and other reagents on enzyme activity were determined at 30 °C in a standard assay medium containing 1 mM each reagent. Effect of NaCl on the activity was determined in a medium containing 0.6 M NaCl. Substrate specificity of  $\beta$ -1,4-mannanase was assessed by measuring activity in assay media containing 0.5% (w/v) linear  $\beta$ -1,4-mannan from *C. fragile*, locust bean gum, tara gum, guar gum, glucomannan, dextran, carboxy methyl cellulose, agarose, and xylan.

### 2.3. SDS-PAGE

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (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.

#### *2.4. Thin-layer chromatography of mannan and mannooligosaccharides*

Mannans and mannooligosaccharides were allowed to react with  $\beta$ -1,4-mannanase and the degradation products were analyzed by thin-layer chromatography (TLC) as follows. Mannans and mannooligosaccharides (M2-M6) were dissolved in 10 mM sodium phosphate (pH 7.0) to make final concentration of 0.5% (w/v) and one unit of  $\beta$ -mannanase (in 25  $\mu$ l of 2 mM sodium phosphate buffer (pH 6.0)) was added to the medium (0.5 ml) and incubated at 30 °C. Then, an aliquot (10  $\mu$ l) of the reaction medium was withdrawn at appropriate time intervals and heated at 100 °C for 2 min to inactivate the enzyme. The products were then spotted on a TLC-60 plate (Merck, Darmstadt, Germany) and developed at room temperature with 1-butanol–acetic acid–water (2:1:1, v:v:v). The sugars fractionated on the plate were detected by heating the plate at 110 °C for 5 min after spraying 10% (v/v) sulfuric acid in ethanol.

#### *2.5. Determination of amino-acid sequence*

The N-terminal amino-acid sequence of  $\beta$ -1,4-mannanase was determined with an ABI Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA). The protein sample was dialyzed against 10% acetonitrile–0.1% trifluoroacetic acid (TFA) and subjected to the sequencer. Internal sequences of  $\beta$ -1,4-mannanase were determined with a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) by using an ABI Proteomics Analyzer 4700 (Applied Biosystems, Foster city, CA, USA). For this purpose, the protein sample was subjected to SDS-PAGE, and target band was excised from the gel, cut into pieces of  $\sim 1 \text{ mm}^3$  and dehydrated with 100% acetonitrile. The gel pieces were dried with centrifugal evaporator and swollen with a reducing agent (10 mM dithiothreitol–25 mM  $\text{NH}_4\text{HCO}_3$ ), washed with 25 mM  $\text{NH}_4\text{HCO}_3$  and alkylated with 55 mM

ICH<sub>2</sub>CONH<sub>2</sub>–25 mM NH<sub>4</sub>HCO<sub>3</sub>. The gel pieces were dehydrated again with 50% acetonitrile–25 mM NH<sub>4</sub>HCO<sub>3</sub>, and the protein in the gel pieces was digested with 0.1 µg/mL trypsin in 25 mM NH<sub>4</sub> HCO<sub>3</sub> at 37 °C for 16 h. Peptide fragments were then extracted from the gel pieces with 50% (v/v) acetonitrile–5% (v/v) TFA and dried with centrifugal evaporator. The peptides were dissolved in 0.1% (v/v) TFA and desalted with ZipTip (MILLIPORE, Billerica, MA, USA). The peptides were finally guanidinated by the method of Wang et al. (2004) and subjected to an ABI 4700 proteomics analyzer (Applied Biosystems). Amino-acid sequences of the peptides were analyzed in a MS/MS mode with DeNovo Explorer software (Applied Biosystems).

#### *2.6. Determination of protein concentration*

Protein concentration was determined by the biuret method (Gornall et al., 1949) or the method of Lowry et al. (1951) using bovine serum albumin as a standard protein.

### **3. Results**

#### *3.1. Purification of a β-1,4-mannanase from A. kurodai*

A β-1,4-mannanase was purified from the crude enzyme of *A. kurodai* as follows. First, the crude enzyme was fractionated with ammonium sulfate and proteins precipitated between 40 and 60% saturation of ammonium sulfate were collected by centrifugation at 10,000×g for 10 min. The precipitates were dissolved in and dialyzed against 40%-saturated ammonium sulfate in 10 mM sodium phosphate buffer (pH 7.0) and applied to a TOYOPEARL Phenyl-650M column (2.5 × 26.5 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted by a stepwise elution with 40%-, 30%-, 10%-, and 0%-saturated ammonium sulfate solution at a flow rate of 15 mL/h. In this chromatography, mannanase activity was detected in the fractions eluted by 10%-saturated ammonium

sulfate. Then, the active fractions, i.e., fractions 82-85 (each contained 15 mL, total 60 mL), were pooled and precipitated with 60% saturation of ammonium sulfate, and collected by centrifugation at 10,000×g for 10 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 7.0), and subjected to a TOYOPEARL CM-650M column (1.5 cm × 20 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted by a linear gradient of 0–0.3 M NaCl (pH 7.0) at a flow rate of 15 mL/h, and the fractions showing mannan-degrading activity, i.e., fractions 75–78 (each contained 5 mL, total 20 mL) were pooled. The pooled fraction was dialyzed against 2 mM sodium phosphate buffer (pH 7.0) and subjected to AKTA-FPLC (GE Healthcare UK Ltd) equipped with a Mono-S 5/50GL column pre-equilibrated with the same buffer. The adsorbed proteins were eluted by a linear gradient with 0–0.5 M NaCl at a flow rate of 60 mL/h and the activity was detected in a single major peak. SDS-PAGE of the active fraction showed a single protein band with an approximate molecular mass of 40,000 Da (Fig. 1). By the above procedure, a mannanase, named AkMan in the present study, was purified 14.5-fold from the crude enzyme at a recovery of 3.3% and the specific activity of 27.4 U/mg (Table 1).

### *3.2. Effects of temperature and pH on AkMan*

AkMan showed an optimal temperature at 55 °C, and exhibited relatively high activity in an acidic pH range, i.e., pH 4.0–7.5 (Fig. 2A and C). AkMan was considerably heat-stable, i.e., temperature at which activity decreased to 50% of the original value by 20-min incubation was 52 °C (Fig. 2B). It should be noted that the activity of AkMan practically unchanged by the incubation at 40 °C for 20 min in the pH range 4–7.5 (Fig. 2D). Thus AkMan was found to be considerably stable especially in acidic pH conditions.

### 3.3. Effects of metal ions and chemical reagents on AkMan

The effects of various metal ions and reagents on the activity of AkMan are summarized in Table 2. Among the metal ions,  $\text{Fe}^{3+}$  caused the strongest inhibition on AkMan, i.e., it decreased the activity to ~30% of the original value. Whereas,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ag}^{+}$  moderately inhibited the activity, i.e., they decreased the activity to 80–50%. On the other hand,  $\text{Co}^{2+}$ ,  $\text{Cu}^{+2}$ ,  $\text{Mn}^{+2}$ , DTT and 2-mercaptoethanol enhanced the activity up to ~50%. The presence of 0.6 M NaCl showed practically no effect on the activity of AkMan, although this enzyme was derived from a marine gastropod.

### 3.4. Substrate preference and mode of action of AkMan

To assess the substrate preference of AkMan, we measured the enzyme activity with various polysaccharide substrates. As shown in Fig. 3, AkMan most preferably degraded a linear  $\beta$ -1,4 mannan from *C. fragile*, then glucomannan, locust bean gum, tara gum, and guar gum in this order. On the other hand, AkMan showed practically no activity toward dextran, carboxymethyl cellulose, agarose, and xylan. These results indicate that AkMan is a specific enzyme to mannan polysaccharides.

The degradation products from both mannan and mannooligosaccharides produced by AkMan were analyzed by TLC. As shown in Fig. 4A, mannotriose (M3) and mannobiose (M2) were produced as major degradation products from the linear  $\beta$ -1,4-mannan from *C. fragile*. Similar products were produced from locust bean gum (galactomannan, mannose:galactose = ~4:1) (Fig. 4B). On the other hand, an oligosaccharide showing the mobility similar to mannotetraose was produced as a major degradation product from tara gum (galactomannan, mannose:galactose = ~3:1) along with mannotriose and mannobiose (Fig. 4C). This tetrasaccharide-like product may be derived from the mannotriose moiety possessing a galactose branch because one galactose branch is known to attach to mannotriose units in tara gum. On the other hand, only trace amounts of oligosaccharides were detected in the

degradation products of guar gum (galactomannan, mannose:galactose = ~2:1) (Fig. 4D). Since one galactose branch is known to attach to mannobiose unit of guar gum, AkMan may not degrade efficiently such a highly branched main chain. This low degree of degradation of guar gum is consistent with the low specific activity of AkMan toward guar gum (Fig. 3). It is noteworthy that AkMan fairly well degraded glucomannan producing various sizes of oligosaccharides (Fig. 3 and 4E). These oligosaccharides showed apparently different mobility in TLC compared with standard manno oligosaccharides. Namely, a sugar in the position between mannose and mannobiose was released from glucomannan (Fig. 4E). It is reasonable to consider that the oligosaccharides released from glucomannan are heterooligosaccharides consisting of mannose and glucose since  $\beta$ -1,4-mannosyl linkages of glucomannan seem to be split by AkMan. Thus, AkMan was shown to be capable of degrading various mannans producing many kinds of oligosaccharides with different structure. The putative split sites for each mannan by AkMan are schematically shown in Fig. 5. The mode of action of AkMan indicates that this enzyme is a typical endo- $\beta$ -1,4-mannanase (EC 3.2.1.78) specifically splitting internal  $\beta$ -1,4-mannosidic linkages of  $\beta$ -mannans.

AkMan was found to be capable of degrading oligosaccharides larger than tetrasaccharides (Fig. 4F). Major degradation products from mannopentaose were mannotriose and mannobiose and that from mannohexaose was mannotriose. Accordingly, the spatial expanse of the substrate binding site of AkMan was considered to be similar to the size of mannopentaose–mannohexaose unit. Accordingly, AkMan seems to recognize mannohexaose unit of mannan and cleave its central  $\beta$ -1,4-mannosyl linkage.

### *3.5. Partial amino-acid sequences of AkMan*

The N-terminal and internal amino acid sequences of AkMan were analyzed by protein sequencer and MALDI-TOF-MS/MS, respectively. Amino-acid sequence of the N-terminal 40 residues of AkMan

showed approximately 55%, 47%, 42% and 40% identity with corresponding regions of *Haliotis*, *Littorina*, *Pomacea* and *Mytilus*  $\beta$ -mannanases, respectively (Fig. 6). In addition, the amino-acid sequence of a lysylendopeptidyl fragment of AkMan, FLNWQADAIK, showed 80% identity to the 223-232 residues of *Haliotis* enzyme and 218-227 residues of *Mytilus* enzyme, respectively. The amino-acid sequences of other two lysylendopeptidyl fragments of AkMan, FHSYSWQ GK and NKYLYQDILR, showed 78% and 50% identities to the 281-289 residues and 213-222 residues of *Haliotis* enzyme, respectively. On the other hand, the sequence TTDPGALVTMGVWNP K of a lysylendopeptidyl fragment, showed 56% and 63% identities to the 233-248 and 228-243 residues of *Haliotis* and *Mytilus* enzymes, respectively. Since *Haliotis* and *Mytilus* enzymes have been classified under the glycosyl hydrolase family 5 (GHF5), we may conclude that AkMan is a member of GHF5 enzymes.

#### 4. Discussion

In the present study, we successfully isolated a mannan-degrading enzyme from the common sea hare *A. kurodai* and identified it as an endo- $\beta$ -1,4 mannanase (EC 3.2.1.78). The molecular mass of AkMan was estimated to be 40,000 Da, which is similar to those of  $\beta$ -1,4-mannanases from *P. insularis* (44,000 Da) (Yamaura and Matsumoto, 1993), *L. brevicula* (42,000 Da) (Yamaura et al., 1996), *M. edulis* (39,216 and 39,265 Da) (Xu et al., 2002a) and *H. discus hannai* (39,627 Da) (Ootsuka et al., 2006). The N-terminal and partial amino-acid sequences of AkMan showed high similarity to those of the above molluscan enzymes belonging to GHF5. Accordingly, AkMan was regarded as a member of GHF5 enzymes.

The optimal temperature of AkMan was shown at 55 °C and temperature that caused a half inactivation of AkMan during 20-min incubation was 52 °C. These temperatures are comparable to those reported with  $\beta$ -1,4-mannanases from *M. edulis* (Xu et al., 2002) and *Bacillus subtilis* NM-39 (Mendoza

et al., 1994). While other molluscan  $\beta$ -1,4-mannanases showed lower optimal temperature and stability than did AkMan. For example, the optimum temperature of *Haliotis* enzyme, HdMan, was 45 °C and the temperature that caused a half inactivation during 30-min incubation was 40 °C (Ootsuka et. al., 2006). The high optimal temperature of AkMan may correlate to the high habitat temperature of *A. kurodai*. Namely, the habitat temperature of *A. kurodai* in summer frequently increases to 25 – 30 °C, which is 12–15 °C higher than that of *H. discus hannai*. The optimal pH of AkMan spanned 4.0-7.5 and the activity was not greatly decreased in the acidic pH range by the incubation at 40 °C for 20 min. These results indicate that AkMan is considerably stable in acidic pH conditions. The stability in the acid pH range seems to be advantageous to AkMan since the pH in stomach lumen of *A. kurodai* is usually less than 6. The optimal pHs for *Pomacea*, *Littorina*, and *Mytilus* enzymes were also in an acidic range, i.e., pH 5.5, pH 6.5, and pH 5.2, respectively. One exception among molluscan  $\beta$ -1,4-mannanases is the *Haliotis* enzyme, HdMan. This enzyme showed an optimal pH at 7.5. Tolerance to wide pH range was also reported in mannanase from *Bacillus* sp. MSJ-5 (Zhang et al., 2009) and *Bacillus subtilis* NM-39 (Mendoza et al., 1994).

AkMan was inhibited to 70% and 50% by  $\text{Fe}^{3+}$  and  $\text{Ag}^+$ ; however, no significant inhibitory effect was found in other metal ions tested. In addition, AkMan was activated by  $\text{Co}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , DTT and 2-mercaptoethanol. Such inhibitory effects of  $\text{Fe}^{3+}$  and  $\text{Ag}^+$  on  $\beta$ -1,4-mannanase have been reported in  $\beta$ -1,4-mannanase from *Bacillus* sp. MSJ-5 (Zhang et al., 2009). Complete inactivation by  $\text{Ag}^+$  and 40–50% inactivation by  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Cu}^{2+}$  were reported in *Haliotis*, *Pomacea* and *Littorina* enzymes (Ootsuka et al., 2006; Yamaura and Matsumoto, 1993; Yamaura et al., 1996). Mannanases from *Streptomyces* sp., *Pseudomonas* sp. PT-5 and *Aeromonas* sp. F-25 were also inactivated by  $\text{Ag}^+$ ,  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$ . Thus, AkMan appeared to be considerably stable against heavy metal ions compared with the other mannanases. The activity of AkMan was not greatly changed by the presence of 0.6 M NaCl. This

indicates that AkMan does not require NaCl for its activity although this enzyme is derived from a marine gastropod.

AkMan could degrade various kinds of mannans but not dextran, carboxymethyl cellulose, agarose and xylan (Fig. 4). Similar results were obtained with the *Haliotis* enzyme (Ootsuka et al., 2006). Among the mannans tested, a  $\beta$ -1,4-mannan from *C. fragile* was most preferably degraded by AkMan, while tara gum and guar gum were not. The difference in the digestibility among the substrates seems to be related to the structural differences in mannans. Namely,  $\beta$ -1,4-mannan from *C. fragile* forms a linear structure, while galactomannan from tara gum and guar gum possesses  $\beta$ -1,6-linked galactose branches in high frequency. The galactose branches in galactomannans may interfere the binding of AkMan to the mannan main chain (see Fig. 5). Major degradation products of  $\beta$ -1,4 mannan from *C. fragile* were mannotriose and mannobiose, while that from tara gum was a mannotetrasaccharide-like sugar. This sugar was considered to be the tetrasaccharide consisting of a mannotriose and a galactose branch. Similar degradation products were produced from ivory nut's mannan by  $\beta$ -1,4-mannanases from *Mytilus*, *T. reesei* and *A. niger* (Xu et al., 2002a, Stalbrand et al., 1993; Ademark et al., 1998). Glucomannan, a linear heteropolysaccharide consisting of  $\beta$ -1,4-linked D-mannose and D-glucose residues, was also readily degraded by AkMan. This indicates that AkMan preferably acts on linear  $\beta$ -1,4-polysaccharides with no branches. The mobility of degradation products from glucomannan in TLC was appreciably different from that of standard manno oligosaccharides. These oligosaccharides were considered to be heterooligosaccharides comprising glucose and mannose residues. AkMan could degrade manno oligosaccharides larger than mannotetraose and produced mannotriose and mannobiose from mannopentaose, while mannotriose from mannohexaose (Fig. 4F). These results suggest that the size of substrate binding site of AkMan is similar to the size of mannopentaose-manno hexaose.

Accordingly, AkMan was considered to bind the mannohexaose unit in the mannan main chain and split the central mannosidic linkages of the mannohexaose unit.

The N-terminal amino-acid sequence of AkMan showed high similarity to those of other molluscan enzymes belonging to GHF5 (Fig. 6). The internal amino-acid sequences of AkMan also showed high similarity to the corresponding sequences of other molluscan enzymes. Thus, AkMan was also regarded as a member of GHF5.

Organisms utilizing mannan as carbon and energy sources are generally known to possess  $\beta$ -1,4-mannanase and  $\beta$ -mannosidase. By the cooperative action of the two enzymes, mannan is completely depolymerized to D-mannose. The D-mannose is metabolized via glycolytic pathway after the isomerization to D-fructose-6-phosphate by phosphomannoisomerase. To date, we have detected  $\beta$ -mannosidase activity in the digestive fluid of *A. kurodai*. Thus, sea hare appeared to be capable of degrading mannan completely to monosaccharide. Biochemical properties of sea hare mannosidase will be published elsewhere. On the other hand, we have reported various kinds of molluscan polysaccharide-degrading enzymes, e.g., alginate lyase, cellulase, and  $\beta$ -1,3-glucanase (Shimizu et al., 2003; Suzuki et al., 2003; Suzuki et al., 2006; Hata et al., 2009; Kumagai and Ojima, 2010). Studies on these enzymes are expected to provide us the useful information about the degradation process of seaweeds' polysaccharides, which are expected as functional polysaccharides for human and a potential biomass in future. In the present study, we could confirm that *A. kurodai* was a good source for the polysaccharide-degrading enzymes and useful for investigation of the mannan-degradation process by mollusks.

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## Legends to figures

**Fig. 1.** SDS-PAGE of different purification steps of  $\beta$ -mannanase from *A. kurodai*. Lane 1, marker protein; lane 2, the crude enzyme; lane 3, the sample after TOYOPEARL Phenyl-650M chromatography; lane 4, the sample after TOYOPEARL CM-650M chromatography; lane 5, the sample after Mono-S 5/50GL column chromatography.

**Fig. 2.** Effects of temperature and pH on AkMan. (A) Temperature dependence of AkMan. Activity was assayed at 15–60 °C using 0.5% locust bean gum in 10 mM sodium phosphate buffer (pH 7.0). (B) Temperature stability of AkMan. AkMan was incubated in 10 mM sodium phosphate (pH 7.0), 0.5 % Locust bean gum and 1U/ml enzyme at 20–60 °C for 20 min. The remaining activity was then assayed at 30 °C. (C) pH dependence of AkMan. Activity was measured at 30 °C for 30 min in reaction mixture adjusted to pH 4.5–6.0 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. (D) pH stability of AkMan. AkMan was 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 6.2) containing 0.5% locust bean gum.

**Fig. 3.** Substrate specificity of AkMan. Activity was measured in a standard reaction mixture containing 0.5% (w/v)  $\beta$ -1, 4-mannan (MA), glucomannan (GM), locust bean gum (LG), tara gum (TG), guar gum (GG), dextran (DX), carboxy methyl cellulose (CM), agarose (AG), and xylan (XL).

**Fig. 4.** Thin-layer chromatography of degradation products from mannans and manno oligosaccharides produced by AkMan. The reaction mixture (0.5 mL) containing 2.5 mg of each substrate in 10 mM

sodium phosphate buffer (pH 7.0) and AkMan (1.1U) was incubated at 30 °C for 0-24 h and 2µl of each reaction product was subjected to TLC plate. Substrates used were (A) β- 1,4 mannan from *C. fragile*, (B) locust bean gum, (C) tara gum, (D) guar gum, (E) glucomannan and (F) mannobiose to mannohexose (M2 – M6) where annooligosaccharides were degraded at 30 °C for 1 hour.

**Fig. 5.** Schematic representation for the degradation of mannans by AkMan. (A) β-1,4 mannan, (B) locust bean gum, (C) tara gum, (D) guar gum, and (E) glucomannan. Arrows indicate the putative split sites of mannans. In case of guar gum (D), the split sites are indicated with gray arrows because AkMan hardly degraded this polysaccharide. M, mannose residues; Gal, galactose residues; and Glc, glucose residues.

**Fig. 6.** Alignment of the N-terminal amino-acid sequences of β-mannanases from *Aplysia*, *Haliotis*, *Mytilus*, *Littorina* and *Pomacea*. Identical, highly conservative, and conservative residues among sequences are indicated by asterisk (\*), colon (:), dot (.), respectively.

**Table 1.**

Summary for the purification of AkMan.

Samples	Total Protein (mg)	Specific activity (U <sup>*c</sup> /mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude <sup>*a</sup>	2191.6	1.9	4159.4	1	100
Phenyl <sup>*b</sup>	388.0	3.1	1196.1	1.6	28.8
CM <sup>*c</sup>	10.4	17.3	179.8	9.1	4.3
Mono-S <sup>*d</sup>	5.0	27.4	137.7	14.5	3.3

<sup>\*a</sup> Crude enzyme after the dialysis against 2 m M sodium phosphate (pH 7.0)

<sup>\*b</sup> Active fraction obtained by TOYOPEARL Phenyl-650M chromatography.

<sup>\*c</sup> Active fraction obtained by TOYOPEARL CM-650M chromatography.

<sup>\*d</sup> AkMan purified by Mono-S chromatography.

<sup>\*e</sup> One unit (U) of mannanase was defined as the amount of enzyme that liberated reducing sugars equivalent to 1.0  $\mu$ mol of D-mannose per minute from 0.5% locust bean gum.

**Table 2.**

Effects of metal ions and reagents on the activity of AkMan.

Reagents	Relative activity (%)
None	100.0
AgNO <sub>3</sub>	47.9
CaCl <sub>2</sub>	86.2
CoCl <sub>2</sub>	106.5
CuSO <sub>4</sub>	114.2
FeCl <sub>3</sub>	30.6
MgCl <sub>2</sub>	83.7
MnCl <sub>2</sub>	126.6
NaCl	94.5
DTT	128.8
2-mercaptoethanol	152.8

Fig. 1.

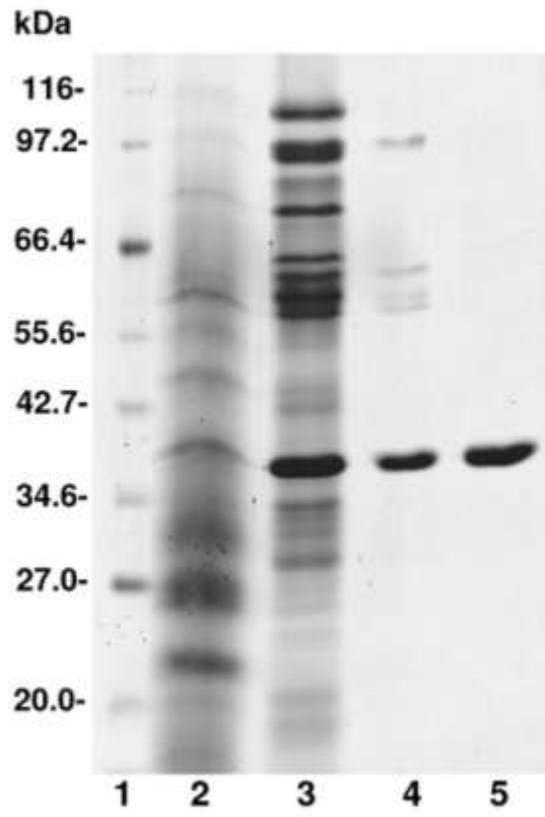


Fig. 2.

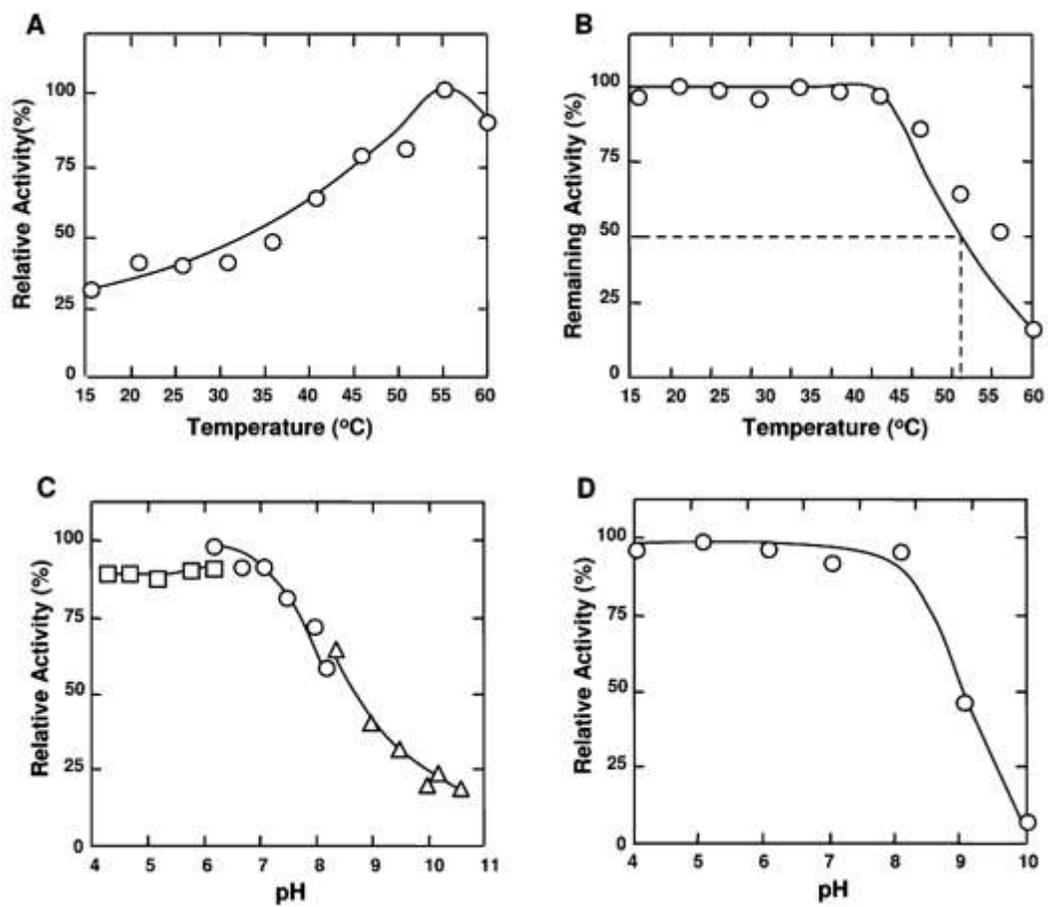


Fig. 3.

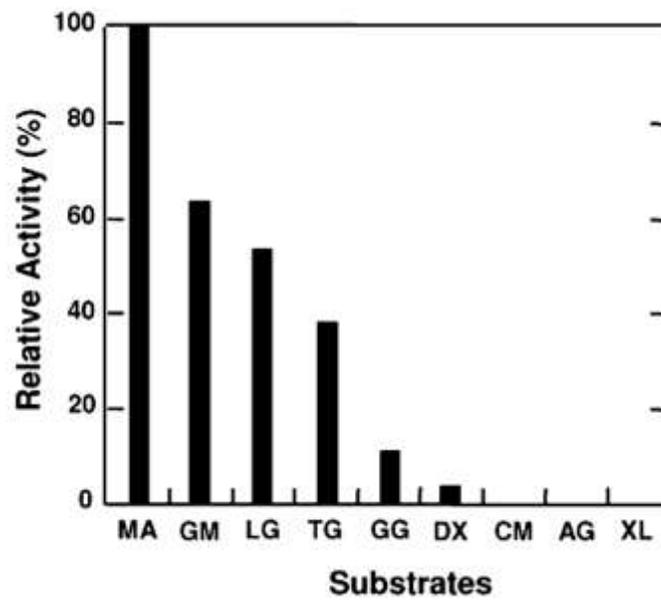


Fig. 4.

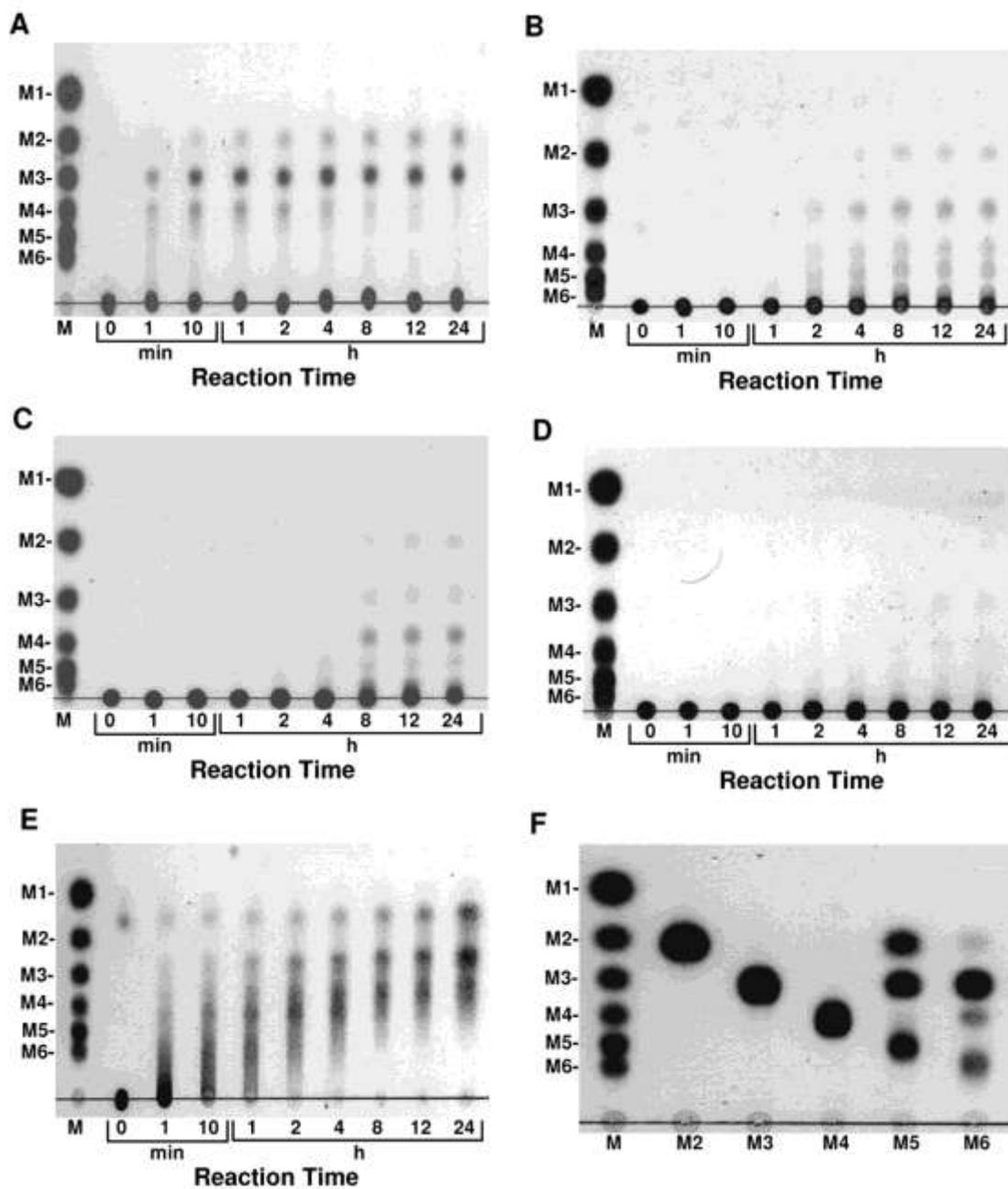


Fig. 5.

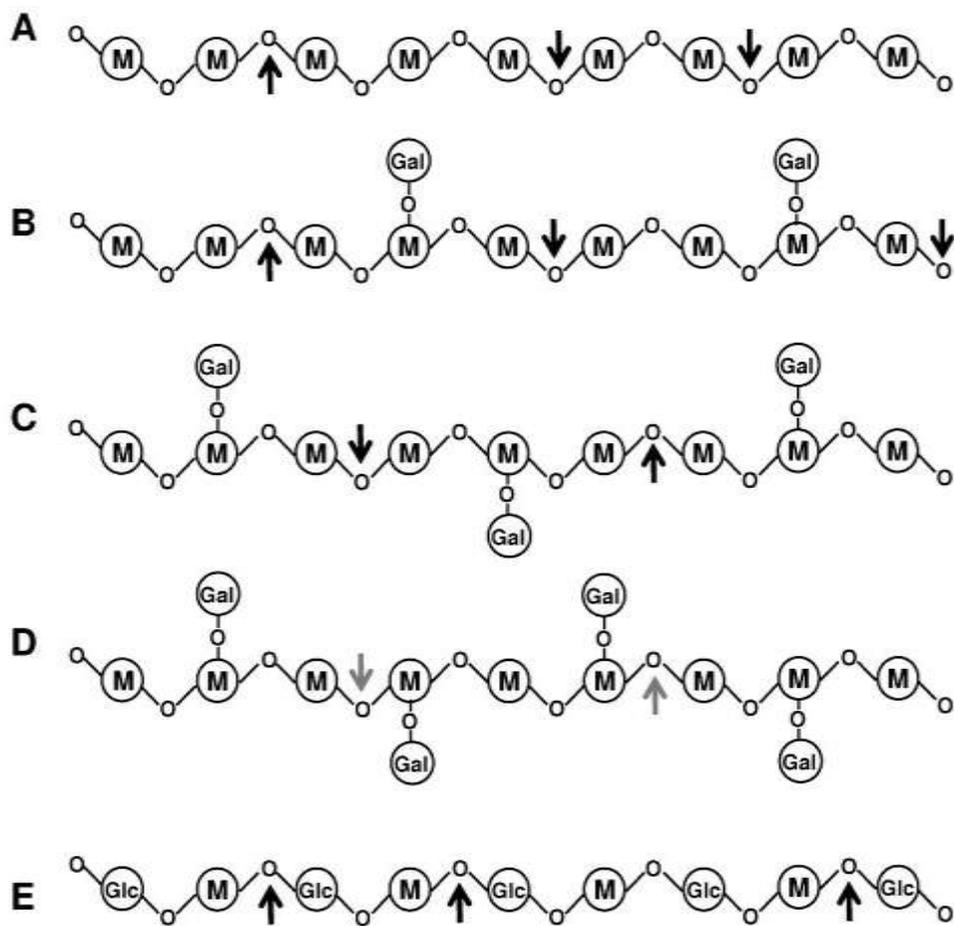


Fig. 6.

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      1          10          20          30          40
      |          |          |          |          |
AkMan  RLHIQNGHFVLN-GQRVFLSGGNLPWMSYAYDFGDGQWQRN
HdMan  DRLSVQGNHFVKG-GQKVFLSGANLAWVQYAYDFGNNHYKG-
Mytilus RLSVSGTNLNYN-GHHIFLSGANQAWVNYARDFGHNQYSKG
Littorina GYLTRSGKHFVHN-GKKVFLSGANQAWVAYG-----
Pomacea GXLRRQGTNIVDSHGKVFSLGANQAWVAYGYD-----
      *  . .  ::  .  *:::*****.*  .*:  *.
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