



Title	Characterization of a $\alpha$ -D-mannosidase from a marine gastropod, <i>Aplysia kurodai</i>
Author(s)	Zahura, Umme Afsari; Rahman, Mohammad Matiur; Inoue, Akira; Ojima, Takao
Citation	Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 162(1-3), 24-33 <a href="https://doi.org/10.1016/j.cbpb.2012.02.003">https://doi.org/10.1016/j.cbpb.2012.02.003</a>
Issue Date	2012-05
Doc URL	<a href="http://hdl.handle.net/2115/49356">http://hdl.handle.net/2115/49356</a>
Type	article (author version)
File Information	CBPB162-1-3_24-33.pdf



[Instructions for use](#)

# Characterization of a $\beta$ -D-mannosidase from a marine gastropod, *Aplysia kurodai*

Umme Afsari Zahura, Mohammad Matiur Rahman, Akira Inoue, and Takao Ojima\*

<sup>1</sup>Laboratory of Marine Biotechnology and Microbiology, Graduate School of Fisheries Sciences,  
Hokkaido University, Minato-cho 3-1-1, Hakodate 041-8611, Japan

\*Corresponding author: Tel: +81 138 40 8800; fax: +81 138 40 8800.

*E-mail address:* ojima@fish.hokudai.ac.jp (T. Ojima)

## Abstract

A  $\beta$ -D-mannosidase (EC 3.2.1.25) with a molecular mass of approximately 100 kDa was purified from the digestive fluid of a marine gastropod *Aplysia kurodai* by ammonium sulfate fractionation followed by column chromatographies on TOYOPEARL Butyl-650 M, TOYOPEARL DEAE-650 M, and Superdex 200 10/300 GL. This enzyme, named AkMnsd in the present study, showed optimal activities at pH 4.5 and 40°C and was stable at the acidic pH range from 2.0 to 6.7 and the temperature below 38°C. The  $K_m$  and  $V_{max}$  values for AkMnsd determined at pH 6.0 and 30°C with *p*-nitrophenyl  $\beta$ -D-mannopyranoside were 0.10 mM and 3.75  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively. AkMnsd degraded various polymer mannans as well as mannoooligosaccharides liberating mannose as a major degradation product. Linear mannan from green alga *Codium fragile* was completely depolymerized by AkMnsd in the presence of AkMan, an endolytic  $\beta$ -mannanase, which we previously isolated from the same animal (Zahura et al., *Comp. Biochem. Physiol.*, B 157, 137-148 (2010)). A cDNA encoding AkMnsd was amplified from the *Aplysia* hepatopancreas cDNA by the PCR using degenerated primers designed on the basis of N-terminal and internal amino-acid sequences of AkMnsd. The cloned AkMnsd cDNA consisted of 2985 bp and encoded an amino-acid sequence of 931 residues with the calculated molecular mass of 101970 Da. The deduced sequence of AkMnsd showed 20-43% amino-acid identity to those of glycoside-hydrolase-family 2 (GHF2)  $\beta$ -mannosidases. The catalytically important amino-acid residues determined in GHF2 enzymes were completely conserved in AkMnsd. Thus, AkMnsd is regarded as a new member of GHF2 mannosidase from marine gastropod.

*Key words:* *Aplysia kurodai*; gastropod;  $\beta$ -mannosidase;  $\beta$ -1,4-mannan; mannoooligosaccharides.

## 1. Introduction

$\beta$ -1,4-Mannan, a major hemicellulosic constituent of plants' tissues, is classified into four types of mannan on the basis of sugar composition and side-chain structure, i.e., linear mannan, galactomannan, glucomannan and galactoglucomannan (Moreira and Filho, 2008). Linear mannan is a homopolysaccharide that comprises a linear chain of 1,4-linked  $\beta$ -D-mannopyranosyl residues containing less than 5% of galactose residues. Linear mannan exists as a structural polysaccharide in fronds of marine algae such as *Porphyra umbilicalis* and *Codium fragile* (Moreira and Filho, 2008), wood and beans in terrestrial plants (Aspinall, 1959), and endosperm of Palmae (Petkowicz et al., 2001). In nature, mannans appear to be consumed mainly by microorganisms which can depolymerize mannans into mannose with mannan-degrading enzymes. In aquatic environment, mannans in algal fronds and their detritus are consumed not only by microorganisms but also by benthic animals such as annelids, arthropods and mollusks. These invertebrates also possess mannan-degrading enzymes and assimilate the mannans as a carbon and energy sources (Chuang and Yang, 1991; Xu et al., 2002a; Song et al., 2008; Ootsuka et al., 2006; Zahura et al., 2010). For complete depolymerization of mannan, at least two types of mannan-degrading enzymes are necessary. One is the  $\beta$ -1,4-mannanase (EC 3.2.1.78, term  $\beta$ -mannanase in the present study) that degrades polymer mannans in an endolytic manner. Another is the  $\beta$ -D-mannosidase (EC 3.2.1.25, term  $\beta$ -mannosidase in the present study) that degrades mannan-oligosaccharides to mannose units. Since  $\beta$ -mannosidase is usually capable of releasing the mannose from the nonreducing terminus of polymer mannans, this enzyme is occasionally called as an exolytic mannanase.

Mannan-degrading enzymes are useful in various industrial processes. For example,  $\beta$ -mannanase improves the clarity of instant coffee and fruit juice, and promotes pulp bleaching in paper industry (Moreira and Filho, 2008). On the other hand,  $\beta$ -mannosidase is useful for

saccharification of mannans from decayed plant materials in a nutritional purpose for farm animals.  $\beta$ -Mannosidase is also useful for the production of biofuels and bioplastics since this enzyme can produce fermentable sugar from hemicellulosic materials (Moreira and Filho, 2008). Since  $\beta$ -mannosidase is important for releasing of mannose residues of N-glycosyl proteins in lysosome, defect of lysosomal  $\beta$ -mannosidase causes accumulation of mannosidic intermediates in cytosol and gives rise to the relating diseases (Neufeld, 1991). Thus,  $\beta$ -mannosidase has been attracting attentions in such medical fields (Kobata, 1993).

Interest in mannan-degrading enzymes from different sources has been increasing in the past decade especially from the viewpoint of biotechnological application (Singh et al., 2003). While many studies have been focusing on the endolytic mannan-degrading enzymes, i.e.,  $\beta$ -mannanases from plants, bacteria, fungi, and invertebrates, not so much attention has been paid to  $\beta$ -mannosidase. To date, characterization and primary structure analysis of  $\beta$ -mannosidase have been achieved in a few enzymes from bacteria (Duffaud et al., 1997; Stoll et al., 2000; Beki et al., 2003), fungi (Takada et al., 1999; Ademark et al., 2001), and mammals (Chen et al., 1995; Leipprandt et al., 1996; Alkhayat et al., 1998; Beccari et al., 2001). On the basis of the hydrophobic cluster analysis for their primary structures, most of these  $\beta$ -mannosidases were classified under glycosyl hydrolase-family 2 (GHF2) (Chen et al., 1995; Alkhayat et al., 1998; Takada et al., 1999; Ademark et al., 2001). Occurrence of  $\beta$ -mannosidase has also been reported in some mollusks (Sugahara et al., 1972; McCleary, 1983; Muramatsu, 1966; Andreotti et al., 2005); however, there appears no report on the primary structure of molluscan  $\beta$ -mannosidase. Thus, the GHF to which the molluscan  $\beta$ -mannosidases belongs has not been revealed.

Recently, we isolated and characterized AkMan, a  $\beta$ -mannanase from a common sea hare *Aplysia kurodai*, and determined its primary structure by cDNA cloning (Zahura et al., 2010 and 2011). *A. kurodai* is known as a typical herbivorous marine mollusk which possesses various kinds

of polysaccharide-degrading enzymes, e.g., alginate lyase (Rahman et al., 2010 and 2011) and laminarinase (Kumagai and Ojima, 2010) in addition to  $\beta$ -mannanase (Zahura et al., 2010 and 2011). These enzymes are considered to play important roles for the degradation of seaweeds' polysaccharides in *A. kurodai* and providing the carbohydrate nutrients for this animal. As for the mannan degradation, AkMan splits the internal  $\beta$ -1,4-mannosyl linkages of the seaweeds'  $\beta$ -mannan producing tri- and disaccharides. But this enzyme could not produce mannose (Zahura et al., 2010). On the other hand, we recently noticed that digestive fluid (crude enzyme) of *A. kurodai* can produce mannose from various mannan substrates. This implied that the digestive fluid of *A. kurodai* contained  $\beta$ -mannosidase.

Therefore, in the present study, we attempted to isolate the  $\beta$ -mannosidase from *A. kurodai*. As a result, a  $\beta$ -mannosidase, named AkMnsd in the present study, was successfully isolated from the digestive fluid. Further, we succeeded to clone the cDNA encoding AkMnsd and analyzed the primary structure of this enzyme. As far as we know, this is the first report on the cDNA cloning and primary structure analysis for  $\beta$ -mannosidase from invertebrates.

## **2. Materials and methods**

### *2.1. Materials*

A synthetic substrate, *p*-nitrophenyl  $\beta$ -D-mannopyranoside, was purchased from Sigma-Aldrich Co. (Tokyo, Japan). Locust bean gum (galactomannan with a molar ratio for mannose:galactose is ~4:1) was kindly supplied by MRC POLYSACCHARIDE Co. Ltd. (Toyama, Japan). Mannooligosaccharides (mannobiose~mannohexaose, M2~M6) were from Megazyme (Bray, Ireland) and the other chemicals with reagent grade were from Wako Pure Chemical Industries Ltd.

(Osaka, Japan). Linear mannan was prepared from *C. fragile* according to the method of Love and Percival (1964). This mannan preparation comprised of approximately 90% of mannose and less than 5 % of galactose and contained no appreciable amount of oligosaccharides. TOYOPEARL DEAE-650 M and TOYOPEARL Butyl-650 M were from Toyo Soda Mfg. Co. (Tokyo, Japan), and Superdex 200 10/300 GL from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England).

Digestive fluid was obtained from the stomach lumen of *A. kurodai* (body length, ~12 cm), which was collected in the summer of 2009 from the shore of Hakodate, Hokkaido, Japan. The digestive fluid from 12 animals (approximately 90 mL) was dialyzed against 2 mM sodium phosphate buffer (pH 7.0) for 5 h and centrifuged at 10000×g for 15 min to remove insoluble materials, and used for the purification of  $\beta$ -mannosidase. To prepare mRNA of *A. kurodai*, hepatopancreas was excised from an *A. kurodai* anesthetized in ice-water. The hepatopancreas was immediately frozen with liquid nitrogen and had been stored at -80°C until RNA extraction. TA PCR cloning kit (pTAC-1) was purchased from Biodynamics (Tokyo, Japan). Oligotex-dT(30), cDNA synthesis kit, 5'- and 3'-Full RACE kits, restriction endonucleases, T4 DNA ligase, and *Escherichia coli* strain DH5 $\alpha$  were purchased from TaKaRa (Tokyo, Japan). AmpliTaq Gold PCR Master Mix and BigDye-Terminator Cycle Sequencing kit were from Applied Biosystems (Foster city, CA, USA). Bacto tryptone, Bacto yeast extract and other reagents used were from Wako Pure Chemicals Industries Ltd.

## 2.2. Purification of AkMnsd from *A. kurodai*

Crude enzyme from *A. kurodai* (approximately 90 mL) was first subjected to ammonium sulfate fractionation. The highest  $\beta$ -mannosidase activity was detected in the fraction precipitated

between 40-70% saturation of ammonium sulfate. This fraction was dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 40% saturation of ammonium sulfate and dialyzed against the same buffer for 24 h. The dialysate was centrifuged at 10000×g for 20 min, and the supernatant was applied to a TOYOPEARL Butyl-650 M column (1.5 cm × 20 cm) pre-equilibrated with the same buffer. The proteins adsorbed to the column was eluted by a stepwise elution system consisting of 40%-, 30%-, 10%- and 0%- saturation of ammonium sulfate (each 375 mL) at a flow rate of 15 mL/h. The eluent was collected as 15 mL fractions.  $\beta$ -Mannosidase activity was detected in the fractions eluted with 10% saturation of ammonium sulfate. The active fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0). A small amount of aggregates formed during dialysis was removed by centrifugation at 10000×g for 10 min, and the supernatant was subjected to TOYOPEARL DEAE-650M column (1.5 cm × 23 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0). Proteins adsorbed to the column were eluted with a linear gradient of 0-0.2 M NaCl at a flow rate of 15 mL/h. Active fractions showing  $\beta$ -mannosidase activity were pooled, dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and lyophilized. The pellets were dissolved in 10 mM sodium phosphate buffer (pH 6.0) containing 0.05 M NaCl, dialyzed against the same buffer and subjected to gel-filtration through Superdex 200 10/300 GL column installed in AKTA-FPLC (GE Healthcare UK Ltd.). Proteins were eluted with 10 mM sodium phosphate buffer (pH 6.0) containing 0.05 M NaCl at a flow rate of 60 mL/h. Active fractions were pooled and subjected again to the same column. The second gel-filtration gave a single peak with  $\beta$ -mannosidase activity and the thus purified protein showed a single band with approximately 100 kDa on SDS-PAGE (Fig. 1). We named this enzyme AkMnsd.

### *2.3. Assay for $\beta$ -mannosidase activity*



$\beta$ -Mannosidase activity was assayed at 30°C in a 1 mL of reaction medium containing 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside, 10 mM sodium citrate buffer (pH 4.5) and an appropriate amount of enzyme. The reaction was terminated in 15 min by the addition of 2 mL of 0.1 M Na<sub>2</sub>CO<sub>3</sub> to the reaction medium and the *p*-nitrophenol produced was determined by measuring the absorbance at 410 nm. One unit of  $\beta$ -mannosidase activity was defined as the amount of enzyme catalyzing the release of 1.0  $\mu$ mol of *p*-nitrophenol per minute under the assay conditions. The average values for triplicate measurements were adopted as activity values.

#### 2.4. SDS-PAGE

SDS-Polyacrylamide gel electrophoresis 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.

#### 2.5. 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 fraction V as a standard protein.

#### 2.6. pH and thermal stabilities of *AkMnsd*

To determine the optimum pH of AkMnsd activity, the following buffers were used to adjust the pH of reaction mixtures; pH 2.0-3.0 with 10 mM glycine-HCl, pH 3.0-6.0 with 10 mM citrate-NaOH, and pH 6.0-8.3 with 10 mM sodium phosphate. To assess pH stability of the enzyme, AkMnsd was first incubated at 40°C for 20 min in 50 mM sodium phosphate buffer adjusted to pH 2.0-9.0, then an aliquot of the incubated medium (usually 0.1 mL) was added to 0.9 mL of reaction mixture containing 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside and 50 mM sodium phosphate buffer (pH 6.0) and the activity was measured at 30°C. The pH for the reaction medium was maintained at around 6 during the reaction. Optimum temperature of the enzyme was assessed by measuring the activity at 15-60°C. Thermal stability was assessed by measuring the activity remaining after the heat treatment at 15-55°C for 20 min.

### 2.7. Enzyme kinetics

To determine the kinetic parameters, i.e., the Michaelis-Menten constant ( $K_m$ ) and the maximal reaction velocity ( $V_{max}$ ), of AkMnsd, the activity was determined with various concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mM) of *p*-nitrophenyl  $\beta$ -D-mannopyranoside. Other reaction conditions were the same as the standard conditions.  $K_m$  and  $V_{max}$  values were estimated from the Lineweaver-Burk plot.

### 2.8. Thin-layer chromatography of degradation products of mannan and manno oligosaccharides

Degradation products of mannan and manno oligosaccharides produced by AkMnsd were analyzed by thin-layer chromatography (TLC) as follows. Linear mannan from *C. fragile* and manno oligosaccharides (M2–M6) were dissolved in 10 mM sodium phosphate buffer (pH 6.0) to

make a final concentration of 0.5% (w/v). To 25  $\mu$ L of each substrate solution, 25  $\mu$ L of  $\beta$ -mannosidase (0.25 units) was added to initiate reaction and the mixture was incubated at 30°C for 24 h. Linear mannan was degraded in 100  $\mu$ L of reaction mixture at 30°C and at an appropriate time interval 10  $\mu$ L of samples were withdrawn from the reaction mixture. The samples were then heated at 100°C for 2 min to terminate the reaction. Two microliters of each reaction mixture were applied to a TLC-60 plate (Merck, Darmstadt, Germany) and developed with 1-butanol-acetic acid-water (2:1:1, v:v:v). The sugars developed on the plate were visualized by heating at 110°C for 15 min after spraying 10% (v/v) sulfuric acid in ethanol.

### *2.9. Determination of cooperative action of AkMnsd and AkMan on mannan substrates*

To examine the cooperative action of  $\beta$ -mannosidase (AkMnsd) and  $\beta$ -mannanase (AkMan), two mannan substrates, i.e., linear mannan and galactomannan were used as substrates. The 0.5 mL of reaction mixture containing 2.5 mg/mL of each substrate in 10 mM sodium phosphate buffer (pH 6.0) and 25  $\mu$ L (0.25 unit) of each enzyme was incubated at 30°C for 0-24 h. An aliquot (10  $\mu$ L) of the reaction mixture was withdrawn at appropriate time intervals and heated at 100°C for 2 min to inactivate the enzymes. The degradation products were analyzed by TLC as described above.

### *2.10. Determination of N-terminal and internal amino-acid sequences*

The N-terminal amino-acid sequence of AkMnsd was determined with an ABI Procise 492 protein sequencer (Applied Biosystems, Foster City, CA, USA). The purified protein was dialyzed against 10% acetonitrile-0.1% trifluoroacetic acid (TFA) and concentrated by a centrifugal evaporator and subjected to the protein sequencer. Internal peptide sequences of AkMnsd 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 purified protein sample was subjected to SDS-PAGE, and the target band was excised from the gel, cut into pieces of  $\sim 1 \text{ mm}^3$ , destained and dehydrated with solution (50% acetonitrile in 25 mM  $\text{NH}_4\text{HCO}_3$ ) and 100% acetonitrile, respectively. The gel pieces were dried with a centrifugal evaporator and swollen with a reducing agent (10 mM dithiothreitol in 25 mM  $\text{NH}_4\text{HCO}_3$ ), washed with 25 mM  $\text{NH}_4\text{HCO}_3$  and alkylated with 55 mM  $\text{ICH}_2\text{CONH}_2$  in 25 mM  $\text{NH}_4\text{HCO}_3$ . The gel pieces were dehydrated again with 50% acetonitrile in 25 mM  $\text{NH}_4\text{HCO}_3$  and dried up completely in a vacuum desiccator. The protein in the gel pieces was then digested with 0.1  $\mu\text{g}/\text{mL}$  trypsin in 25 mM  $\text{NH}_4\text{HCO}_3$  at  $37^\circ\text{C}$  for 16 h. Peptide fragments were then extracted from the gel pieces with 50% (v/v) acetonitrile in 5% (v/v) TFA and dried with a centrifugal evaporator. The peptides were dissolved in 0.1% (v/v) TFA and desalted with ZipTip (MILLIPORE, Billerica, MA, USA). The peptides bound to ZipTip 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 by DeNovo Explorer software in a MS/MS mode (Applied Biosystems). Homology searches for the amino-acid sequences on the public databases were performed with the FASTA and BLAST programs (<http://fasta.ddbj.nig.ac.jp/top-j.html>, <http://blast.ddbj.nig.ac.jp/top-j.html>) provided by DNA Data Bank of Japan.

### *2.11. cDNA cloning and nucleotide sequencing*

Total RNA of *A. kurodai* was extracted from 1g of hepatopancreas, which had been stored at  $-80^\circ\text{C}$  after quick freezing with liquid nitrogen, by the guanidinium thiocyanate-phenol method (Chomczynski and Sacchi, 1987) and mRNA was selected from the total RNA with Oligotex-dT(30)

according to the manufacturer's protocol. Hepatopancreas cDNA was synthesized with the mRNA by using a TaKaRa cDNA synthesis kit and random hexanucleotide primers. cDNAs encoding AkMnsd were amplified from the hepatopancreas cDNA by PCR using degenerated primers synthesized on the basis of partial amino-acid sequences of AkMnsd. The PCR was performed in 20  $\mu$ L of reaction mixture containing 50 mM KCl, 15 mM Tris-HCl (pH 8.0), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 2.5 mM MgCl<sub>2</sub>, and 5 pmol/ $\mu$ L primers, 1 ng/ $\mu$ L template DNA, and 0.5 units AmpliTaq Gold DNA polymerase. A successive reaction at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s was repeated for 40 cycles with Thermal Cycler Dice mini (TaKaRa, Tokyo, Japan). The size of the amplified cDNA was estimated by 1.0% agarose-gel electrophoresis and cloned with TA PCR cloning kit (pTAC-1) (Invitrogen, CA, USA) and *E. coli* DH5 $\alpha$ . The transformants were grown in 2  $\times$  YT medium supplemented by 50  $\mu$ g/mL ampicillin at 37°C for 14 h by shaking at 150 rpm/min. The plasmids extracted from the transformants were subjected to the sequence analysis with BigDye-Terminator Cycle Sequencing kit and ABI 3130xl 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 according to the manufacturers' protocols.

### **3. Results**

#### *3.1. General properties of the purified AkMnsd*

AkMnsd was purified from the digestive fluid of *A. kurodai* through ammonium sulfate fractionation followed by the conventional column chromatographies. In the final gel-filtration through Superdex 200 10/300 GL, AkMnsd eluted as a single peak and showed a single protein band

with an approximate molecular mass of 100 kDa on SDS-PAGE (Fig. 1). AkMnsd was purified 23.7-fold from the crude enzyme at a recovery of 2.7% and the specific activity of 25.8 U/mg (Table1).

The optimum pH and temperature of AkMnsd were observed at around pH 4.5 and 40°C, respectively (Fig. 2A and C). AkMnsd was stable at acidic pH conditions, i.e., 50% of the original activity was retained after the incubation at pH 2.0-6.7 and 40°C for 20 min (Fig. 2B). The temperature that caused a half inactivation of AkMnsd during 20 min incubation was 38°C (Fig. 2D).

Kinetic parameters,  $K_m$  and  $V_{max}$ , of AkMnsd to the substrate *p*-nitrophenyl  $\beta$ -D-mannopyranoside were estimated to be 0.10 mM and 3.75 U/mg, respectively.

### 3.2. Degradation of manno oligosaccharides and linear mannan by AkMnsd

To examine the activity of AkMnsd toward oligosaccharide substrates, manno oligosaccharides (M2~M6) were degraded by AkMnsd and the reaction products were analyzed by TLC (Fig. 3). AkMnsd could degrade all the oligosaccharides tested to mannose (Fig. 3A). It should be noted that the degradation of manno oligosaccharides appeared to take place exolytically. Namely, M6 was first degraded to M5 which was one mannose unit smaller than M6 and the produced M5 was then degraded to M4 which was one mannose unit smaller than M5 (Fig. 3B). This stepwise degradation seemed to continue until most of the original substrate was converted to mannose. The exolytic action of AkMnsd was also seen in a linear  $\beta$ -mannan substrate (Fig. 3C). Thus, AkMnsd produced only mannose from the linear  $\beta$ -mannan without any intermediate oligosaccharides. These analyses for the degradation products strongly suggested that AkMnsd acted on the substrates in an exolytic manner.

### 3.3. Cooperative action of AkMnsd and AkMan on mannan

As mentioned in the “Introduction” part, AkMan, an endolytic  $\beta$ -mannanase of *A. kurodai*, may degrade seaweeds’ mannan together with AkMnsd to produce mannose efficiently. As shown in Fig. 4A, the linear mannan was readily degraded by the action of AkMan and AkMnsd to mannotriose and mannobiose in the early stage of the reaction, and then converted to mannose in the later stage. The amount of mannose produced by the two enzymes was considerably higher than that produced by AkMnsd alone (see Fig. 3C). On the other hand, AkMan and AkMnsd degraded locust bean gum (galactomannan) to produce not only mannose but also hexasaccharide, pentasaccharide and trisaccharide (Fig. 4B). These oligosaccharides may be derived from galactose-branching regions. Namely, the galactose branches of locust bean gum may inhibit the actions of AkMan and AkMnsd and make the branching regions undigested.

#### 3.4. cDNA cloning and primary structure of AkMnsd

The N-terminal amino-acid sequence of 40 residues of AkMnsd was determined as YERVPLDGQLNWMLSEASAGVNI PASVPGSMY TALLEKNL by the protein sequencer. This sequence showed 22.5% amino-acid identity with the GHF2  $\beta$ -mannosidases from *Homo sapiens* (Alkhatat et al., 1998; DDBJ accession number, U60337) and *Caenorhabditis elegans* (DDBJ accession number, Z78540), 27.5% identity with the enzymes from *Aspergillus aculeatus* (DDBJ accession number, AB015509). The sequences of tryptic fragments of AkMnsd (Table 2) also showed 10-80% identities with the sequences of GHF2  $\beta$ -mannosidases from various organisms. Such sequence similarity of AkMnsd to GHF2 enzymes suggested that AkMnsd also belongs to GHF2.

cDNAs encoding AkMnsd were amplified by PCR. First, AkMnsd-cDNA-1, consisting of 1791 bp and encoding an amino-acid sequence of 597 residues, was amplified by PCR with the degenerated forward and reverse primers, AkMnsdFw and AkMnsdRv, which were designed on the basis of N-terminal and an internal amino-acid sequences of AkMnsd, respectively (Table 3). Then 3'-RACE and 5'-RACE PCRs were performed with the specific primers shown in Table 3 and cDNA-3RACE (1077bp) and cDNA-5RACE (437bp) were amplified, respectively. By overlapping the nucleotide sequences of cDNA-5RACE, AkMnsd-cDNA-1 and cDNA-3RACE, in this order, the nucleotide sequence of total 2985 bp including the complete translational region of AkMnsd was determined (Fig. 5). In this sequence, the translational initiation codon, ATG, was found to locate in the nucleotide positions from 107 to 109 and termination codon, TAG, from 2900 to 2902. Accordingly, the amino-acid sequence of 931 residues was deduced from the translational region of 2793 bp which spans 107-2899th nucleotides. In the 3'-terminal region, a putative polyadenylation signal sequence, AATGAA, and a poly (A+) tail-like sequence were found. The N-terminal region of 14 residues except for the initiation Met, i.e., FWFHVSLLIATGIS, was predicted as the signal peptide for secretion by the Signal P3.0 software (<http://www.cbs.dtu.dk/services/SignalP/>). The sequence of 12 amino acids, QWMLKTNNLVSS, which locates just after the signal peptide, was regarded as a propeptide-like region of this enzyme since this region was absent in the native AkMnsd protein. Therefore, the mature AkMnsd was considered to consist of 904 amino-acid residues with the calculated molecular mass of 101970 Da. This molecular mass is well consistent with the molecular mass, 100 kDa, estimated by SDS-PAGE (see Fig. 1). All the internal peptide sequences of T-1 to T-5 (Table 2) are seen in the deduced sequence (Fig. 5). Thus, the cDNA was concluded to be of AkMnsd. (the nucleotide and the deduced amino-acid sequences are available from the DNA Data Bank of Japan with the accession number [AB685732](#)).

We compared the deduced amino-acid sequence of AkMnsd with those of several  $\beta$ -



mannosidases belonging to glycoside hydrolase family 2 (GHF2) (Fig. 6). The amino-acid sequence of AkMnsd showed 43%, 24% and 20% identities with  $\beta$ -mannosidases from *H. sapiens* (Alkhayat et al., 1998), *A. aculeatus* (Takada et al., 1999) and *C. elegans* (DDBJ accession number, **Z78540**), respectively. The two glutamate residues which are known as the common catalytic residues of GHF2 were conserved as Glu467 and Glu563 in the deduced sequence of AkMnsd (Fig. 6). Accordingly, AkMnsd from *A. kurodai* was concluded as an enzyme belonging to GHF2.

#### 4. Discussion

During the purification of  $\beta$ -1,4-mannanase from *A. kurodai* (Zahura et al., 2010), we noticed the occurrence of  $\beta$ -mannosidase(s) in the crude enzyme preparation. In the present study, we successfully purified the  $\beta$ -mannosidase, AkMnsd, from the crude enzyme. The molecular mass of AkMnsd was estimated to be 100 kDa by SDS-PAGE. Since the molecular masses of  $\beta$ -mannosidases from other organisms were in a range of 94-135 kDa, e.g., *Helix pomatia*, 94 kDa (McCleary, 1983); *Aplysia fasciata*, 130 kDa (Andreotti et al., 2005); *Aspergillus niger*, 135 kDa (Ademark et al., 1999); *Thermobifida fusca* TM51, 94 kDa (Beki et al., 2003); *Aspergillus aculeatus* No.F-50, 104 kDa (Takada et al., 1999); *Trichoderma reesei*, 105 $\pm$ 5 kDa (Kulminskaya et al., 1999); and *A. niger*, 102.335 kDa (Ademark et al., 2001), the molecular mass of AkMnsd was found to be comparable with those of other  $\beta$ -mannosidases.

The optimum pH and temperature of AkMnsd was pH 4.5 and 40°C, respectively (Fig. 2A and 2C). A  $\beta$ -mannosidase from *A. fasciata* showed similar optimal pH, i.e., pH 4.5 (Andreotti et al., 2005), whereas it showed higher optimal temperature 45°C, which is 5 degrees higher than that of AkMnsd. On the other hand, the optimum pH and temperature of  $\beta$ -mannosidase from a terrestrial mollusk, *H. pomatia*, were at pH 4.0 and 55°C (McCleary, 1983). Most of fungal  $\beta$ -mannosidases

showed optimal activity in acidic pHs, i.e., pH 2.4-5.0 for *A. niger* (Ademark et al., 1999) and pH 3.5 for *T. reesei* (Kulminskaya et al., 1999). AkMnsd was considerably stable at acidic pH conditions. Namely, the activity retained 50% after the incubation at 40°C for 20 min in the pH range of 2.0-6.7 (Fig. 2B).  $\beta$ -Mannosidases from some organisms also showed similar pH stability in acidic conditions. For example, *T. reesei* enzyme was stable in a pH range of 3.5-6.0 (Kulminskaya et al., 1999) and *A. niger* enzyme was stable at pH 4.0-6.0 (Ademark et al., 1999). However, thermal stability of AkMnsd in the acidic pH range was somewhat lower compared with  $\beta$ -mannosidases from *A. niger* (Bouquelet et al., 1978) and *Thermotoga neapolitana* 5068 (Duffaud et al., 1997). Thus, *A. niger* enzyme was not inactivated by the incubation at 50°C for 24 h and *T. neapolitana* 5068 enzyme showed half-lives of 18 h at 85°C, 42 min at 90°C, and 2 min at 98°C.

The  $K_m$  and  $V_{max}$  values for AkMnsd were 0.10 mM and 3.75 U/mg, respectively. More or less similar  $K_m$  and  $V_{max}$  values were found in  $\beta$ -mannosidases from *T. fusca* TM51 ( $K_m=180 \mu\text{M}$  and  $V_{max}=5.96$ ; Beki et al., 2003) and *T. reesei* ( $K_m=0.12 \text{ mM}$ ; Kulminskaya et al., 1999). On the other hand, significantly higher  $K_m$  and  $V_{max}$  values were determined for the  $\beta$ -mannosidase from *A. fasciata*, i.e., 2.4 mM and 50.3  $\mu\text{mol}/\text{min}/\text{mg}$ , respectively (Andreotti et al., 2005). Significantly higher  $K_m$  values i.e., 1.43 mM and 6.5 mM were determined for other molluscan  $\beta$ -mannosidase from *H. pomatia* (McCleary, 1983) and *A. fulica* (Sugahara et al., 1972), respectively. The  $K_m$  values for the  $\beta$ -mannosidases from *H. sapiens* (Noeske and Mersmann, 1983) and *A. niger* (Bouquelet et al., 1978) were 2.2 mM and 0.46 mM, respectively. Thus, the  $K_m$  value for AkMnsd was considered to be more similar to those of fungal enzymes than those of other molluscan enzymes.

AkMnsd could degrade various sizes of mannoooligosaccharides (M2~M6) (Fig. 3A). This indicated that AkMnsd is a typical  $\beta$ -D-mannosidase. When mannohexaose (M6) was degraded by AkMnsd, mannose unit was released along with the stepwise decreasing of the substrate size from M6 to M2 (Fig. 3B). This result indicated that AkMnsd exolytically cleaved the  $\beta$ -1,4-mannosidic

bond of mannoooligosaccharides. Such an exolytic action has been found in the mannosidases from a terrestrial snail, *H. pomatia* (McCleary, 1983) and *A. niger* (Ademark et al., 1999) and these enzymes were shown to act on the non-reducing terminus of substrates.

AkMnsd was capable of degrading not only mannoooligosaccharides but also polymer mannan. When linear mannan was degraded by AkMnsd, mannose was produced as a major product (Fig. 3C). This result indicated that AkMnsd acted on polymer substrate with an exolytic manner. On the other hand, when linear mannan was degraded by both AkMnsd and AkMan, linear mannan was more efficiently degraded to mannose accompanying the production of some intermediate oligosaccharides (Fig. 4A). This indicated that AkMnsd and AkMan could act on the seaweed's mannan cooperatively in the digestive fluid of *A. kurodai* to produce mannose efficiently. On the other hand, when locust bean gum, a galactomannan, was degraded by the two enzymes, some oligosaccharides were remained to be undigested (Fig. 4B). The apparent sizes of the oligosaccharide were tri-, penta- and hexasaccharides. This suggests that AkMnsd and AkMan hardly degrade the branching region of galactomannan. Consequently, the remained tri-, penta- and hexaoligosaccharides were considered to be oligosaccharides possessing galactose branches. The higher order structures of such oligosaccharides will be determined in future.

The nucleotide sequence of AkMnsd cDNA which encodes the amino-acid sequence of 931 residues was determined. The deduced sequence comprised the initiation Met, the putative signal peptide of 14 residues, the propeptide-like region of 12 residues, and mature enzyme region of 904 residues. The calculated molecular mass of the mature enzyme region was 101970 Da, which was well consistent with the molecular mass of native AkMnsd (approximately 100 kDa) estimated by SDS-PAGE. The amino-acid sequence of the mature enzyme region showed 20~43% identities with those of GHF2  $\beta$ -mannosidases from animal and fungus sources (Alkhatat et al., 1998; Takada et al., 1999). GHF2 includes various types of polysaccharide-degrading enzymes such as  $\beta$ -galactosidase,

$\beta$ -glucuronidase, exo- $\beta$ -glucosaminidase (Llanillo et al., 1977; Diez and Cabezas, 1978; Nogawa et al., 1998). Such GHF2 enzymes, two glutamate residues are known as catalytically important. According to the three-dimensional structures of  $\beta$ -glucuronidases from human (Jain et al., 1996) and *E. coli* (Jacobson et al., 1994), the two glutamate residues i.e., Glu457 and Glu554 for *H. sapiens* and Glu461 and Glu537 for *E. coli* were found to participate in the catalytic action. These Glu residues are conserved in  $\beta$ -mannosidases from *H. sapiens*, *A. aculeatus* and *C. elegans* (Fig. 6) and also in AkMnsd as Glu467 and Glu563. One glutamate residue (Glu519) in a  $\beta$ -mannosidase from *C. fimi* was revealed to be the catalytic nucleophile (Stoll et al., 2000). These conserved Glu residue in AkMnsd indicated that this enzyme is a new member of GHF2  $\beta$ -mannosidase.

### **Acknowledgment**

This study was supported in part by the grants for project research (Development of fundamental technology for analysis and evaluation of functional agricultural products and functional foods), Ministry of Agriculture, Forestry and Fisheries, Japan. The authors appreciate Mr. Hiroyuki Tanaka for his assistance in mass spectrometry.

### **References**

- Ademark, P., Lundqvist, J., Hagglund, P., Tenkanen, M., Torto, N., Tjerneld, F., Stalbrand, H., 1999. Hydrolytic properties of a  $\beta$ -mannosidase purified from *Aspergillus niger*. *J. Biotechnol.* 75, 281-289.
- Ademark, P., de Vries, R.P., Hagglund, P., Stalbrand, H., Visser, J., 2001. Cloning and characterization of *Aspergillus niger* genes encoding an  $\alpha$ -galactosidase and a  $\beta$ -mannosidase involved in galactomannan degradation. *Eur. J. Biochem.* 268, 2982-2990.

- Alkhatat, A.H., Kraemer, S.A., Leipprandt, J.R., Macek, M., Kleijer, W.J., Friderici, K.H., 1998. Human  $\beta$ -mannosidase cDNA characterization and first identification of a mutation associated with human  $\beta$ -mannosidosis. *Hum. Mol. Genet.* 7, 75-83.
- Andreotti, G., Giordano, A., Tramice, A., Mollo, E., Trincone, A., 2005. Purification and characterization of a  $\beta$ -mannosidase from the marine anaspidean *Aplysia fasciata*. *J. Biotechnol.* 119, 26-35.
- Aspinall, G.O., 1959. Structural chemistry of the hemicelluloses. *Adv. Carbohydr. Chem.* 14, 429-468.
- Beccari, T., Bibi, L., Stinchi, S., Stirling, J.L., Orlacchio, A., 2001. Mouse beta-mannosidase: cDNA cloning, expression, and chromosomal localization. *Biosci. Rep.* 21, 315-323.
- Bouquelet, S., Spik, G., Monteuil, J., 1978. Properties of a  $\beta$ -mannosidase from *Aspergillus niger*. *Biochim. Biophys. Acta.* 522, 521-530.
- Béki, E., Nagy, I., Vanderleyden, J., Jager, S., Kiss, L., Fülöp, L., Hornok, L., Kukolya, J., 2003. Cloning and Heterologous Expression of a  $\beta$ -D-mannosidase (EC 3.2.1.25)-Encoding gene from *Thermobifida fusca* TM51. *Appl. Environ. Microbiol.* 1944-1952.
- Chen, H., Leipprandt, J.R., Traviss, C.E., Sopher, B.L., Jones, M.Z., Cavanagh, K.T., Friderici, K.H., 1995. Molecular cloning and characterization of bovine  $\beta$ -mannosidase. *J. Biol. Chem.* 270, 3841-3848.
- Chomczynski, P., Sacchi, N., 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
- Chuang, N.N., Yang, B.C., 1991. Purification and properties of a beta-mannosidase from shrimp (*Penaeus japonicus*) hepatopancreas. *Comp. Biochem. Physiol. B.* 98, 627-630.
- Diez, T., Cabezas, J.A., 1978. Properties of two molecular forms of  $\beta$ -glucuronidase from the mollusc *Littorina littorea* L. *Eur. J. Biochem.* 93, 301-311.

- Duffaud, G.D., McCutchen, C.M., Leduc, P., Parker, K.N., Kelly, R.M., 1997. Purification and characterization of extremely thermostable beta-mannanase, beta-mannosidase, and alpha-galactosidase from hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. *Appl. Environ. Microbiol.* 63,169-177.
- Gornall, A.G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177, 751-766.
- Jacobson, R.H., Zhang, X.-J., Dubose, R.F., Matthews, B.W., 1994. Three-dimensional structure of  $\beta$ -galactosidase from *E. coli*. *Nature* 369, 761-766.
- Jain, S., Drendel, W.B., Chen, Z., Mathews, F.S., Sly, W.S., Grubb, J.H., 1996. Structure of human  $\beta$ -glucuronidase reveals candidate lysosomal targeting and active-site motifs. *Nat. Struct. Biol.* 3, 375-381.
- Kobata, A., 1993. Glycobiology: an expanding research area in carbohydrate chemistry. *Acc. Chem. Res.* 26, 319-324.
- Kulminskaya, A.A., Eneiskaya, E.V., Ludmila, S., Isaeva-Ivanova, L.S., Savel'ev, A.N., Sidorenko, I.A., Shabalin, K.A., Golubev, A.M., Neustroev, K.N., 1999. Enzymatic activity and  $\beta$ -galactomannan binding property of  $\beta$ -mannosidase from *Trichoderma reesei*. *Enzyme Microb. Technol.* 25, 372-377.
- Kumagai, Y., Ojima, T., 2010. Isolation and characterization of two types of beta-1,3-glucanases from the common sea hare *Aplysia kurodai*. *Comp. Biochem. Physiol. B* 155, 138-144.
- Leipprandt, J.R., Kraemer, S.A., Haithcock, B.E., Chen, H., Dyme, J.L., Cavanagh, K.T., Friderici, K.H., Jones, M.Z., 1996. Caprine  $\beta$ -mannosidase: sequencing and characterization of the cDNA and identification of the molecular defect of caprine  $\beta$ -mannosidosis. *Genomics* 37, 51-56.
- Llanillo, M., Perez, N., Cabezas, J.A., 1977.  $\beta$ -Galactosidase and  $\beta$ -glucosidase activities of the same enzyme from rabbit liver. *Int. J. Biochem.* 8, 557-564.

- Love, J., Percival, E., 1964. The polysaccharides of green seaweed *Codium fragile*. Part III. A  $\beta$ -linked mannan. J. Chem. Soc. 3345-3350.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
- McCleary, B.V., 1983.  $\beta$ -D-Mannosidase from *Helix pomatia*. Carbohydr. Res. 111, 297-310.
- Moreira, L.R., Filho, E.X., 2008. An overview of mannan structure and mannan-degrading enzyme systems. Appl. Microbiol. Biotechnol. 79, 165-178.
- Muramatsu, T., 1966. Purification of  $\alpha$ -D-mannosidase and  $\beta$ -D-mannosidase from marine gastropods. Arch. Biochem. Biophys. 115, 427-429.
- Neufeld, E.F., 1991. Lysosomal storage diseases. Annu. Rev. Biochem. 60, 257-280.
- Noeske, C., Mersmann, G., 1983.  $\beta$ -D-Mannosidase from human placenta: properties and partial purification. Hoppe-Seyler's Z. Physiol. Chem. 364, 1645-1651.
- Nogawa, M., Takahashi, H., Kashiwagi, A., Ohshima, K., Okada, H., Morikawa, Y., 1998. Purification and characterization of exo- $\beta$ -D-glucosaminidase from a cellulolytic fungus, *Trichoderma reesei* PC-3-7. Appl. Environ. Microbiol. 64, 890-895.
- Ootsuka, S., Saga, N., Suzuki, K., Inoue, A., Ojima, T., 2006. Isolation and cloning of an endo- $\beta$ -1,4-mannanase from Pacific abalone *Haliotis discus hannai*. J. Biotechnol. 125, 269-280.
- Petkowicz, C.L.O., Reicher, F., Chanzy, H., Taravel, F.R., Vuong, R., 2001. Linear mannan in the endosperm of *Schizolobium amazonicum*. Carbohydr. Polym. 44, 107-112.
- Porzio, M.A., Pearson, A.M., 1977. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biochim. Biophys. Acta. 490, 27-34.
- Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2010. Isolation and characterization of two alginate lyase isozymes, AkAly28 and AkAly33, from the common sea hare *Aplysia kurodai*. Comp. Biochem. Physiol. B 157, 317-325.

- Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2011. cDNA cloning of an alginate lyase from a marine gastropod *Aplysia kurodai* and assessment of catalytic important residues of this enzyme. *Biochimie* 93, 1720-1730.
- Singh, S., Madlala, A.M., prior, B.A., 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS. Microbiol. Rev.* 27, 3-16.
- Song, J.M., Nam, K.W., Kang, S.G., Kim, C.G., Kwon, S.T., Lee, Y.H., 2008. Molecular cloning and characterization of a novel cold-active  $\beta$ -1,4-D-mannanase from the antarctic springtail, *Cryptopygus antarcticus*. *Comp. Biochem. Physiol. B.* 151, 32-40.
- Stoll, D., He, S., Withers, S.G., Warren, R.A.J., 2000. Identification of glu-519 as the catalytic nucleophile in  $\beta$ -mannosidase 2A from *Cellulomonas fimi*. *Biochem. J.* 351, 833-838.
- Sugahara, K., Okumura, T., Yamashina, I., 1972. Purification of  $\beta$ -mannosidase from a snail, *Achatina fulica* and its action on glycopeptides. *Biochim. Biophys. Acta.* 268, 448-496.
- Takada, G., Kawaguchi, T., Kaga, T., Sumitani, J., Arai, M., 1999. Cloning and sequencing of  $\beta$ -mannosidase gene from *Aspergillus aculeatus* no. F-50. *Biosci. Biotechnol. Biochem.* 63, 206-209.
- Wang, D., Kalb, S.R., Cotter, R.J., 2004. Improved procedures for N-terminal sulfonation of peptides for matrix-assisted laser desorption/ionization post-source decay peptide sequencing. *Rapid Commun. Mass Spectrom.* 18, 96-102.
- Xu, B., Hägglund, P., Stalbrand, H., Janson, J.C., 2002a. Endo- $\beta$ -1,4-mannanases from blue mussel, *Mytilus edulis*: purification, characterization and mode of action. *J. Biotechnol.* 92, 267-277.
- Zahura, U.A., Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2010. An endo- $\beta$ -1,4-mannanase, AkMan, from the common sea hare *Aplysia kurodai*. *Comp. Biochem. Physiol. B* 157, 137-143.
- Zahura, U.A., Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2011. cDNA cloning and bacterial expression of an endo- $\beta$ -1,4-mannanase, AkMan, from *Aplysia kurodai*. *Comp. Biochem.*



Physiol. B 159, 227-235.

## Legends to figures

**Fig. 1.** The second gel-filtration of AkMnsd through Superdex 200 10/300 GL. The AkMnsd fraction obtained in the first gel-filtration through a Superdex 200 10/300 GL was subjected to the second gel-filtration. Fractions indicated by the horizontal solid bar were pooled. SDS-PAGE for  $\beta$ -mannosidase preparations from *A. kurodai* is shown in the inset. Lane 1, marker protein; lane 2, the crude enzyme; Lane 3, the sample after TOYOPEARL Butyl-650 M; Lane 4, the sample after TOYOPEARL DEAE-650 M; Lane 5, AkMnsd purified by the second gel-filtration through Superdex 200 10/30.

**Fig. 2.** Effects of pH and temperature on AkMnsd. (A) pH dependence of AkMnsd. Activity was assayed at 30°C in reaction mixtures containing 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside adjusted to pH 2.0-3.0 with 10 mM glycine-HCl (■), pH 3.0-6.0 with 10 mM citrate-NaOH (●) and pH 6.0-8.3 with 10 mM sodium phosphate (▲). (B) pH stability of AkMnsd. AkMnsd was incubated at 40°C for 20 min in 50 mM sodium phosphate buffer (pH 2.0-9.0). The remaining activity was assayed at 30°C in a medium containing 50 mM sodium phosphate buffer pH 6.0 and 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside. (C) Temperature dependence of AkMnsd. Activity was assayed at 15-60°C in a reaction mixture containing 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside in 10 mM sodium phosphate buffer (pH 6.0). (D) Thermal stability of AkMnsd. The remaining activity was measured after the heat treatment at 15-55 °C for 20 min.

**Fig. 3.** Thin-layer chromatography for degradation products of mannoooligosaccharides and linear mannan produced by AkMnsd. (A) Degradation of mannose (M1) and mannoooligosaccharides (mannobiose~mannohexaose; M2~M6). “+E” indicates the sample after the enzyme degradation for 24 h. Mk, marker sugars; M1~M6. (B) Degradation of M6. (C) Degradation of linear mannan. The reaction mixture containing 0.5% substrate and 25  $\mu$ L of purified enzyme (0.25 unit) was mixed and allowed to react at 30°C for 0-24 h and 2  $\mu$ L of each reaction product was subjected to TLC.

**Fig. 4.** Thin-layer chromatography for degradation products of different mannans produced by the action of AkMnsd and AkMan. (A) Linear mannan degraded by AkMan and AkMnsd. (B) Locust bean gum degraded by AkMan and AkMnsd. The reaction mixture (0.5 mL) containing 2.5 mg of each substrate in 10 mM sodium phosphate buffer (pH 6.0) and 25  $\mu$ L (0.25 unit) of each enzyme was allowed to react at 30°C for 0-24 h and 2  $\mu$ L of each reaction product was subjected to TLC. Hexasaccharide, pentasaccharide and trisaccharide remained undigested are indicated with “hex”, “pen” and “tri”, respectively.

**Fig. 5.** Nucleotide and deduced amino acid sequence of AkMnsd. The translational initiation codon ATG, termination codon TAG, and a putative polyadenylation signal AATGAA are boxed. A putative signal peptide is indicated by a dotted underline. The N-terminal and the internal sequences of T-1, T-2, T-3, T-4 and T-5 are indicated with lines under the amino-acid sequence.

**Fig. 6.** Comparison of the amino-acid sequence of *A. kurodai* (AkMnsd) with GHF2  $\beta$ -mannosidases. Sequences were cited from *C. elegans* (DDBJ accession number, Z78540), *H. sapiens* (DDBJ

accession number, **U60337**), and *A. aculeatus* (DDBJ accession number, **AB015509**). Identical, highly conservative and conservative residues are indicated by asterisks (\*), colon (:), and dot (.) respectively. The Glu residues predicted to participate in catalytic action are boxed.

**Table 1.** Summary of purification of AkMnsd.

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U <sup>a</sup> /mg)	Yield (%)	Purification (fold)
Crude <sup>b</sup>	730.36	795.66	1.09	100	1
Butyl <sup>c</sup>	23.08	205.76	8.92	25.86	8.18
DEAE <sup>d</sup>	12.79	142.85	11.17	17.95	10.24
Gelfiltration <sup>e</sup>	2.21	48.15	21.80	6.05	20.00
Gelfiltration <sup>f</sup>	0.85	21.71	25.79	2.72	23.66

<sup>a</sup> One unit of  $\beta$ -mannosidase activity was defined as the amount of enzyme that releases of 1.0  $\mu$ mol of *p*-nitrophenol per minute from 2.5 mM *p*-nitrophenyl  $\beta$ -D-mannopyranoside.

<sup>b</sup> Crude enzyme after the dialysis against 2 mM sodium phosphate buffer (pH 6.0).

<sup>c</sup> Active fractions obtained by TOYOPEARL Butyl-650 M chromatography.

<sup>d</sup> Active fractions obtained by TOYOPEARL DEAE-650 M chromatography.

<sup>e</sup> Active fractions obtained by the first gel filtration through Superdex 200 10/300 GL.

<sup>f</sup> AkMnsd purified by the second gel filtration through Superdex 200 10/300 GL.

**Table 2.** N-terminal and internal amino-acid sequences of AkMnsd

---

Peptide <sup>a</sup>	Sequence
N-terminal	YERVPLDGQLNWMLSEASAGVNI PASVPGSMYTALLEKNL
T-1	EGDNSIVVGF
T-2	INGLPVFLKGSNWIPADNFQER
T-3	TDYIYLTQINQAMSM
T-4	HAQDVSDTLAAINK
T-5	TSTSWLFLSYPK

---

<sup>a</sup> T-1–T-5, tryptic fragments.

**Table 3.** Primers used for amplification of AkMnsd-cDNA

	Primer names	Sequences <sup>a, b</sup>
AkMnsd-cDNA-1	AkMnsdFw	5'-GARGCNTTYGCNNGGTNAAYATHCC-3' ( EASAGVNIP)
	AkMnsdRv	5'-CATNSWCATNGCYTGRTTDTATYTGNGT-3' ( TQINQAMSM)
cDNA-3RACE	AkMnsd(3)Fw	5' -GGCTCTGCACATAGACTTGTCC - 3'
	3Adapt	5' -CTGATCTAGAGGTACCGGATCC - 3'
cDNA-5RACE	AkMnsd(5)Fw	5' -AGAAGTCCAGCTACGAGATCC - 3'
	AkMnsd(5)Rv	5' -ACAGACCAGCAGCACCTTGC - 3'

<sup>a</sup> R, adenine or guanine; N, adenine or guanine or cytosine or thymine; Y, cytosine or thymine; H, adenine or cytosine or thymine; S, cytosine or guanine; W, adenine or thymine; and D, adenine or guanine or thymine.

<sup>b</sup> Amino-acid sequences used for designing the degenerated primers are in the parentheses.

**Fig. 1**

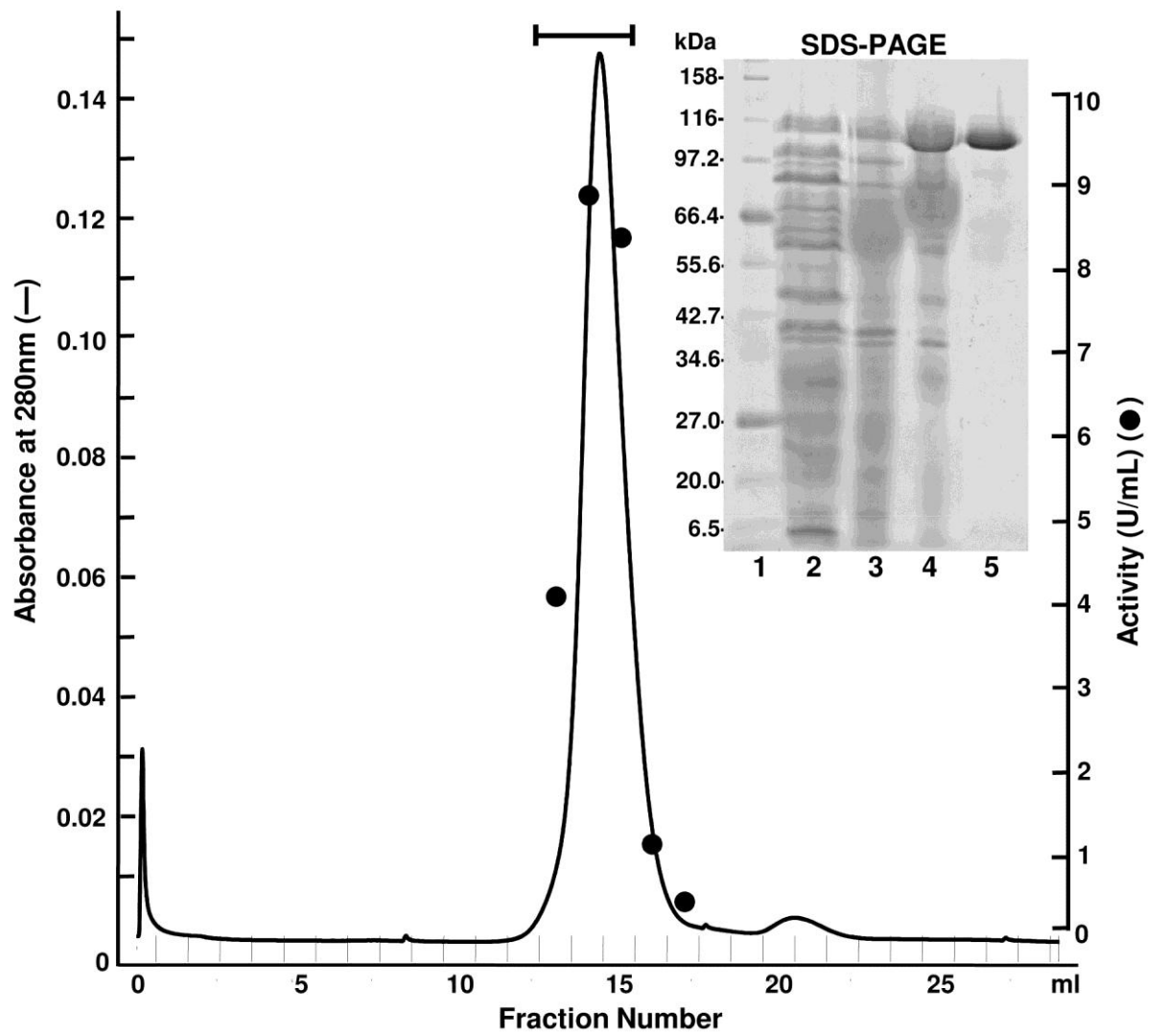




Fig. 2

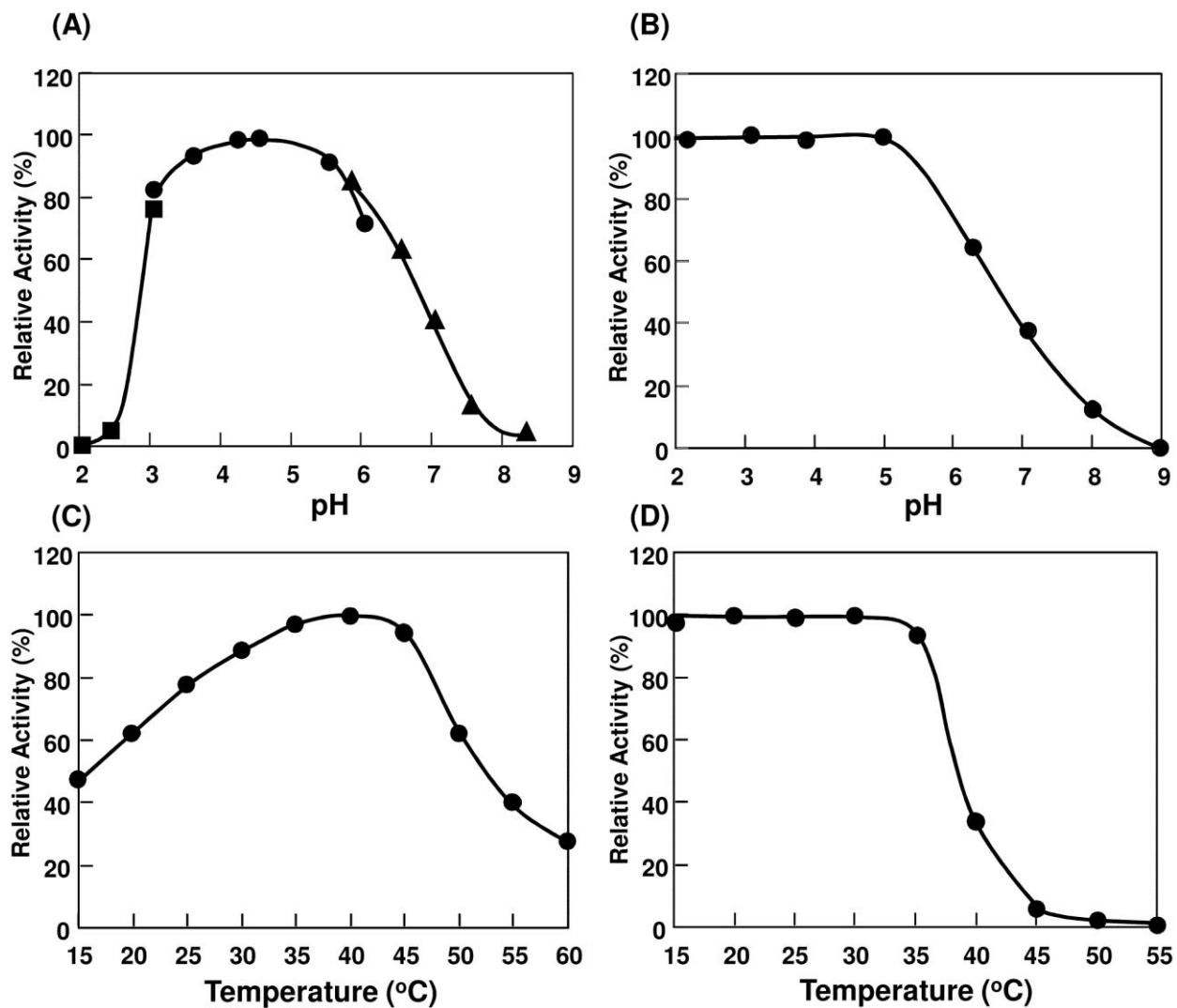


Fig. 3

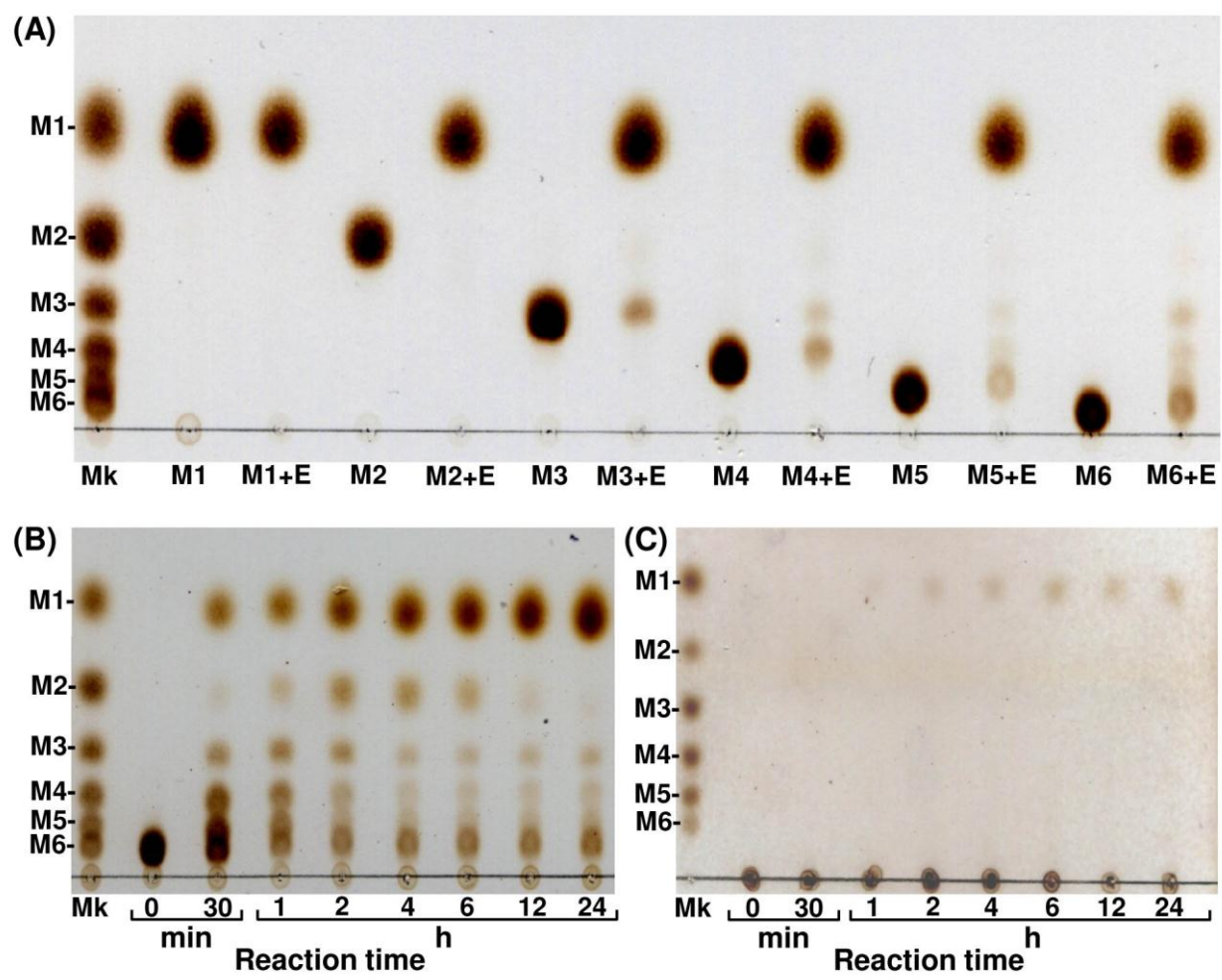
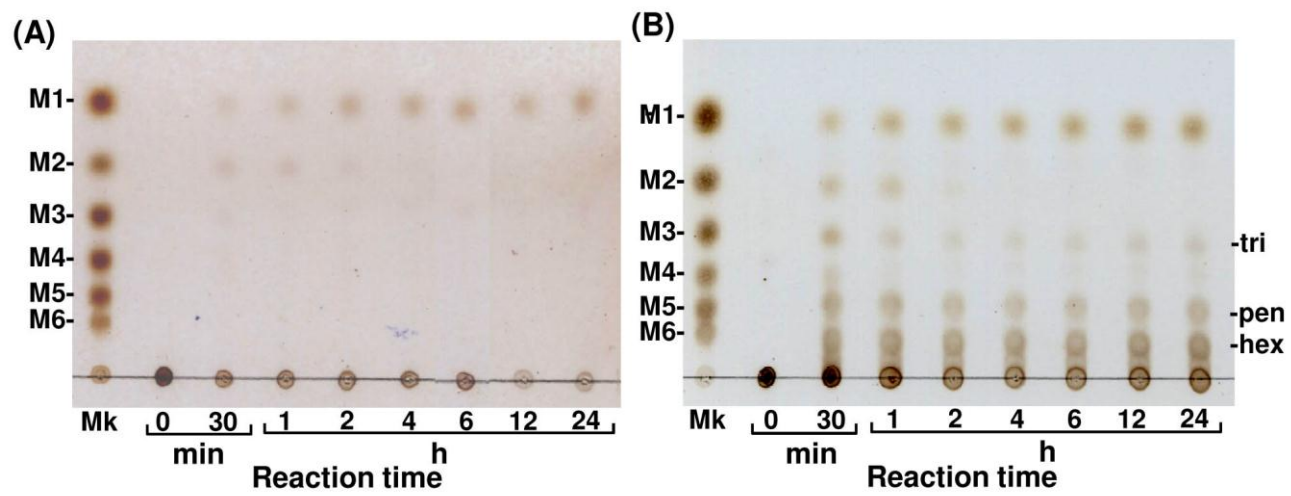


Fig. 4



**Fig. 5**

AGAAGTCCAGCTACGAGATCCGCCCACTGTCCACCCCGGTCAGCAAGGCAGTTCACAAGGTGCGCTGGCAGCAAGAACAACAGACTGCGGCATA 100  
GGCAACATGTTTGGTCCAGCTCTCGCTTCTGATGCTACTGGCATTTACAGTGGATGCTCAAACGAATAATTTGGTCTGCTTTATGAACGGTTC 200  
M E W E H V S L L L I A T G L S Q W M L K T N N L V S S Y E R V 31  
CTCTCGATGGCCAGCTTAATGGATGTTGTTCAGAGGCACTGCAAGGTGTGAATATACCGCGCTGTGCCCGGATCCATGTACACGGCTCTGTTGAAAA 300  
P L D G O L N W M L S E A S A G V N I P A S V P G S M Y T A L L E K 65  
N-terminus  
GAACCTCATCCAGACCCTCTCTATCGGACAACGACAACAATATGCTGGTGGTAACTCGGACTGGACGTACAACAGGAAGTTCACAGTGTCCAAA 400  
N L I Q D P L Y R D N D N K Y A W L G N S D W T Y N R N F T V S K 98  
TCTGTGGCTGAGTCCAGCAAGTGTCTGGTCTGTGAGGGGCTGGACACAGTGGCAGTCTGGTCAACGGAAAGGCAGTGGGCGACACAGACAACA 500  
S V A E S S K V L L V C E G L D T V A S V V V N G K A V G D T D N 131  
TGTTTGTCCCGCATGCCTTTGGCTCACCGGGATCGTGAAGGAAGGTGACAACAGTATTGTCGTAGGCTTCCGGTCAGCGGTGCTTGGAGCAGGAGCG 600  
M F V R H A F D V T G I V K E G D N S I V V G F R S A V L E A Q H F E R 165  
CGACCAGAAGTCCAGCTACGAGATCCGCCCAACTGTCACCCCGGTCACGCAAGGCCAGTGTACAGTGAACAGTGAAGGAAGGAGCAGTGTAGCTTC 700  
D Q K S S Y E I P P N C P P P V Q Q G Q C H V N Q L R K E Q C S F 198  
AGCTGGAGTGGGACCCTCCTTCCACACAGGGCATCTGGAAGCCAATCTA TCTGAATGCGTTTAGTACCGCGTAGTGCAACAGTCTCCGCTATAC 800  
S W D W G P S F P T Q G I W K P I Y L N A F S T A V V Q R V S A I 231  
CTAAGAAAGTGAAGTGGCAGTGGCAGGTGGACGTGATGTAATCTTTGATGTCACAGGGTTTAACAGAGGTCAAGGTCAAGTGGACGTACC 900  
P K K V S G Q W Q V D V D V I F D V N E G L T E V K G Q V Q V D V P 265  
GGCCTTGAAGTGAAGTGGTTCAGGACGTGACAGTTCCTCCAGAGCAGGGCTAAGGTGCTACTCAAAGTGCCTGGCCAGGATGTGCTGCTG 1000  
A L N L T L V Q D V T G I V K S S C T Q S R A K V V L K V P L A R D T G L L 298  
TGGTGGCCAAACGGTTACGGTCTCCAGAACCTGTACAGATGTCCTGACCTTACCTCTGGCTCTGACGTCTCAGCCGCTCTCAAGGTGGCTTCA 1100  
W W P N G Y G L Q N L Y Q M S V T F T S G S D V S S R S F K V G F 331  
GGACAGTGGAGCTGATCCAGACCCCGTCTTCCGACTCTAAGAAAGGCCTGACCCTTCTACTTCCGCATCAACGGTCTCCCGGTGCTCTGAAAGGCTC 1200  
R T V E L I Q D P V S S D S K K G L T F Y F R I N G L P V F L K G S 365  
CAACTGGATCCCGCCGACAACCTCCAGAGGCATCAACAGAGACAGACTGAGCTACCTGCTGCTGAGTCAACGGCCACTGTGGGCAATTAACCTCAAGC 1300  
N W I P A D N F O E R I T R D R L S Y L L Q S T A T V G I N S M R 398  
T-2  
GTGTGGGCGGTGGGTTGACGAGTCAAGATTTCTACGAACTGTGTGATGAGTTTGGTATCATGGTGTGGCAAGCCTCATGTTGAGTGTGCTCTTT 1400  
V W G G G V Y E S E D F Y E L C D E F G I M V W Q D L M F S V A L 431  
ACCCGACCTACGATGGCTTCCCTCAGTCCGTTGGCCACAGAGATCAGACAACAGGTCACACGCTCAACACACACCGTCCATCATTCTGTGGGCTGGCAA 1500  
Y P T Y D G F L Q S V A T E I R Q Q V Q R L K H H P S I I L W A G N 465  
CAACGAGAACGAGAAGGTCGCGCCGAACTGGTCAAAAACAGACAACCTTACCGCTCTATTACAACGATTATGTGAAGTCTACGTCAACACCATC 1600  
N E N E K G L R Q N W F K T Q N N F T L Y Y N D Y V K L Y V T T I 498  
AAACCCATAGTAAACGCGAGGACGACAGCCGTGAGTACCTTCTCCTCAGTCCCTTCAAAGGCAAGAGAGTGAAGAAAGGGTTATGTAGCCAGGAGC 1700  
K P I V N A E D D S R E Y L L S S P S N G K E S E K E G Y V A K E 531  
CTTGGAGTGAAGTCTATGGAGCATTATTTCTACGAAACCTGTGGATCAGTGGAACCCGCGCTGTTCGCGTGTCCCGCATGAGCCTCAAGTACGG 1800  
P W S E L Y G D I H F Y E N V D F Y E L C D E F G I M V W Q D L M F S V A L 565  
CATACAGGCTGGTCAACAACAGAGTCTGGCCTCAGTATTCGCCCAGAGGACTACAGCATGGACGCGCCATGATAGGACACAGGCATCATTAT 1900  
I Q G W C N N E S L A S V F A P E D Y S M D A P M I G H R Q H H Y 598  
AACGGCAACGCTGAAATGGCAGCAGAAGTGGCTCTGCACATAGACTTGTCCACCTTATCGGATCCTGTGCTCAAGTTCACCGACTACATCTACCTTACAC 2000  
N G N A E M A A E V A L H I D L S T S S D P V V K F T D Y I Y L T 631  
AGATCAACAGCCATGTCATGCTACCCAGAGCAGCACTACAGGCGTTCAGTCCGACTGCTCTCTGATGCCGCGGACTACCATGGGCGCCCT 2100  
Q I N O A M S M R T Q S E H Y R R F Q S R L L S D G R G L T M G A L 665  
T-3  
CTACTGGCAGCTCAACGACATCTGGCAGGCTCCCACTGGGCTCCATGACTATGAGGCGCGTGGAAAGTGTGCAATTACTACGCCAGCACTTCTTC 2200  
Y W Q L N D I W Q A P T W A S I D Y E G R W K M L H Y Y A Q H F F 698  
CAGCCACCCCTGGTGTCCGCTGACGACAAACACCCCTCAACGCTTTCATGGCGTGGATGGGTCGCCAGTCTGGAGCGGACGCAAAATTTGA 2300  
Q P T L V S P Y L Q D N N T L N V F M A V D G V P S L E R R D N L 731  
CAGGTGAAGTGAATTTGAGCCATGAGCGATATTGACGACATCAAGAAGAGCAGTATACACGCAACAGCAGTCCGACACTTTGGCAGCCATTAAACA 2400  
T G E L R F E P M S D I D D I K K S S I H A O D V S D T L A A I N K 765  
AGCAACTCCGCTGTTTGACGGTACCATGTACAATTACCGCAGTTTCAACCCCTCAAGTCTGGAATGTTGATTATTCATGTAACAACAGCGCCGAC 2500  
A T S G R L T V T M Y N Y A S F T P L K S W N V D Y S M N T T A D 798  
TCTGTGTACACGGCAGTGTGAGCCAGCTCGTGTGAGCGAGCCAACTGTGTGTCAGGCAAACTGTTTCTTACACTTCTCCACAGCAGACAAAGTAACG 2600  
S V Y T A S V Q L V S E A N C V S R D N C F L H F S T A A D K S N 831  
CCGTGACCTCCACTTGGCTTTTCTCTTACCCAAAGTATCCAACCTGCGCTCGAGCTCAAATCAAGGTGTCGAGTCAAGGACATCTCTCCACG 2700  
A V T S T S W L E L S Y P K Y S N L P R A Q I K V V A V R N A I S P R 865  
T-4  
AGTTTGGAAAGTGGACATCTCCTCTGATGCTTTGCTCTGTACTGCTGGCTATCTGCTGGGACGCTTGGGCAAGTCTCGGATAACGGCTTTCACCTT 2800  
V L E V D I S S D A F A L Y V W L S A G D V L G R F S D N G F H L 898  
CACACCCGACAGCCAGGTCAGTTCATGCGCGGACGATGTGACGGCTGCAAGCTGCAAGGAAATCTCAAAGTAAAAGTTTGTCCGATGTCCAA 2899  
H T P T A T V K F Y A A D D V T A A K L Q G N L K V K S L S D V Q 931  
TACGAAGATGATAATAGAGTGGAGTTCGTTGAACCGAACCGTGTATCCGAACAGCGGTAATGAAAGAAAAA 2985

\*

Fig. 6

<i>A. kurodai</i>	MFWFHVSLLIATGISQWMLKTNINVSSYERV--PLDQLNWMLSEAAGVNI PASVPGSMY TALEKNLI QDPLR DNDNKYAWLGN	85
<i>C. elegans</i>	MRTSLVCLFWLLFQLHTTGHYNTLVNLAG-----NWEFSSSNKT VNGTGT VPGDIYSDL YASGI I DNP LFGENH LNLKWI AE	78
<i>H. sapiens</i>	MRLHLLLLALCG----AGTTAAELSY S-----LRG--NWS I CNGNGS LELPGA VPGCVH SALFQGLI QDS YR FN DNLHRVSL	75
<i>A. aculeatus</i>	MRALPTTATTLLGVLF FPSASRSQYVRDLG---TEQWTLSSATLNRTV PAQ FPSQVHMDL LREGII DEP YND LND FNLRWIAD	80
	:: . . . . . : * . . . . . . . . * . . . : * . . . : * . . : * . .	
<i>A. kurodai</i>	SDWTYNRNFTVSKSVAESSKVLVCEGLD TVASVVNGKAVGDTDNMVF RHAFDVTGI VKEGDN SIVVGFSAVLEAQERDQKSS--	170
<i>C. elegans</i>	DDWTYSRKFR I DLDDTVGAFLEI ESVDTIATV YVNGQKVLH SRNQFL PYHVNVDI IALGENDIT IKFKSSV KYAEKRADEYKKI	164
<i>H. sapiens</i>	DNWTYSKFEKIPFEISKQKVNLLILEGVDTVSKI LFNVT IGE TDNMFNRYS FDI TNVVRD-VN SIE LRFQSAVL YAAQSKAHTX-	160
<i>A. aculeatus</i>	ANWTYTSKGI EGLGEDYES-TWL VFDGLDTFASI SFCGQFVGATDNQFRQYMFVSS I LKACPE EPTLGI QFGSAPNIVDAI AQDP-	165
	:*** . . . . . * . . :*** . . . . . : : * * . . . . . : . . . : . . . . .	
<i>A. kurodai</i>	--YEI PPNCP PPVQGGQCHVN--QLRKEQCS FSWDWG PSFPTQGIWKP-----IY LNAFSTAVVQR--VSAIPKKSQGWQ	240
<i>C. elegans</i>	FGHS LPPDCN PDI YHGEGHQ N--PIRKAQYS FAWDWG PSFPTVGIPTST-----ITINI YRGQYHDFNWKTRFAHGKWKVA	238
<i>H. sapiens</i>	--YQVPPDCP PLVQKGEGHVN--FVRKEQCS FSWDWG PSFPTQGIWKD-----VR IEAYNI CHLN YFTFSPI YDKSAQEW N	232
<i>A. aculeatus</i>	----SPTWPEGVQIT YEY PNRWFMRKE QSDFGWDWG PAFAPAGPWKPGV YVQLKQAA PVY VRNTDLDIYRLGQIN YLPDQTPQPVW	248
	. * . . : : * . . . * . . . . . * . . . . . : . . . . . : . . . . .	
<i>A. kurodai</i>	VDVDVIFD VNEGLTEVKGQVQVDVPALMLTLVQDVTV SSSQSRAKV LKVLPLARDVLLWWPNGYGLQNL YQMSVTF TSGSDVS--SR	325
<i>C. elegans</i>	FEFDTFHYGARTI EYSVQI PELGKESD YR LSA TKSLQT RSKNIMSL S I P MEHE PERWV PNMGEQKLY DVV VSMGGQVKK---	321
<i>H. sapiens</i>	LEIESTF DVVS-SKPVGGQVIXAIPKLQ TQQTYS IELQPGKRI VELFVN I SKNIT VETWVPHGHGNTGY NMTVLFELD GGLNI-EK	317
<i>A. aculeatus</i>	VNASLDY LGS LPENPSMAI EVKDLQSGE ILSRPL TNITV TEGSVT GVTVLE GVD PKLWVP QGLGDQNL YNV TISV TDGNQSVAEV	335
	. . . . . : : : . . . . . : . . . . . : : : * * * . . . . . : . . . . .	
<i>A. kurodai</i>	SFKVGFRTVLELIQDPVSSDSK-K---GLTFYFR INGLPVFLKGSNWI PADNFQ-ERI TRDRLS YLLQSTATVGINSMRVG GGVYE	406
<i>C. elegans</i>	--KIGFKTVELVQDLIDPKKPEK---GRNFYK INDEPV FLKGTNWI P VSMFRS DRENI AKTE FL LDSVAEVGMNAIRVWGGGFYE	402
<i>H. sapiens</i>	SAKVYFR TVELIEEPIKGS P-----GLSFYK INGFPI FLKGSNWI PADS FQ-DRVTSEL LRLLL QSVVDMNMTLRVWGGGIYE	396
<i>A. aculeatus</i>	TKRTGFRTIFLNQRNI TDAQLAQGI APGANWHFE VNGHEFYAKGSN LIPDCFW-TRVTEDTMTLRF DAVVAGNQNMLRVWS SGA YL	421
	. * : * : * : * : * : * . . . . . * . . . . . * . . . . . * . . . . . * . . . . . * . . . . .	
<i>A. kurodai</i>	SEDFYELCDEFGIMVWQDLMFVALYPT YDGF LQSVATEI RQOVQRLKHHPS ILLWAGNNHNEKGLRQNWFKTQN-NFTL YN DYVK	492
<i>C. elegans</i>	SNHFY YASKKGI LVWQDLMFACALYPTTEE FIQNAEEVSYNVDR ISQHTSVIV FSGNNHNEAAIRGHWWKASNY TESQVKDYVL	489
<i>H. sapiens</i>	QDEFYELCDELGMVWQDFMFACALYPTDQGF LDSVTAEVAYQIKR LKS HPS I I I WSGNNHNEEALMNMWYH I SFTDRP IYI KDYVT	483
<i>A. aculeatus</i>	HDYIYDLADEKGI LLSSEFQFS DALYPTDDA FLENVAE VVYVRRVNHHPS LALWAGNNHIES LMLLLV EAADPE SYPFVGEY EK	508
	: * * : . . . : * * : * * * : * * : * * * : * * : * * * : * * : * * * : * * : * * * : * * : * * *	
<i>A. kurodai</i>	LYVTTIKPIVNAEDDSREYLLSPSNG-----KESEKEGYVAKE PWSELYGDI HFYEN--VVDQWN PAVFRVPRMASEYGI QGWCN	571
<i>C. elegans</i>	LYQR-LAKIAK VAPT IPFIMS SPSNG-----VETEEEGGVSKN PYDVR YGDI HYYNE-FVNLWR DDT YLT PRCASEYGVQSYPM	567
<i>H. sapiens</i>	LYVKNIRELV LAGDKSRPFITS SPTNG-----AETVAEAWV SQNPNS NYFGVDY HFYDY-ISDCWNWKV FPKARFAS EYGVQSWPS	562
<i>A. aculeatus</i>	MYISLFL PLVYENTRS ISYSPS STTEGY LDI DLSAPV PMAERY SNTTEGEY YGDT DHYNYDASI AFDYGT YPVGRFANE FGFHSMPS	595
	: * : *	
<i>A. kurodai</i>	NESLASVFAP-EDYSMDP MIGHRHQHHYNGNAEMAAE VALHIDLSTSS-----DPVVKFTDYIYLQ TINQAMSMRTQS	643
<i>C. elegans</i>	KETMLNWINESDWEY TSKAMFHRQHHPGGI ATNLLMIFQHLP IPAECSKS VSDVPS CKYISSASYMSRLAYFSQVHQSI ALKQTQ	653
<i>H. sapiens</i>	FSTLEKVSST-EDWSFN SFLHRQHHEGGNKQMLYQAGLHFKLPQST-----DPLRTFKDTIYLQTMVQAQC VKTET	634
<i>A. aculeatus</i>	LQTWQALTD PADLTFNSSV MLRNHY PAGGLMTDN YHNTVARHGRND PGR-----AGL LPPDAQH SVRPRGQLRLV PRDPA	673
	. . . . . * . . . . . * : * * : . . . . . : . . . . . : . . . . . : . . . . .	
<i>A. kurodai</i>	EHYRFRQSRLSDGRGLTMGAL YWQ LND IWQAPT WASIDY EGR----WKMLHYAQHFQ PTLVSPYLDNNTLNVFMAV DGVPSL	725
<i>C. elegans</i>	LHYRFRNTT TNEGLN TCMAMYQLND VWAAPT WSTIDFEQN----WKMAHYEARFFF SNAVYS FADETD FN LKVFLLNDNPYL	735
<i>H. sapiens</i>	EFYRRSRSEI VDQ-QGHTMGAL YWQLND IWQAPTS WAS LEYGGK----WKMLHYFAQN FFA PLLPVGFENEN----TFYIYGVS DL	710
<i>A. aculeatus</i>	LPGGQLQVTVN PVLPAQQAART PARVPV LAARGH LAGALVGGDRVRRPLEGPHYVARD IYK PVI VSPFWNYTTGALDIYVTS DLWTA	760
	: : . . . . . : : : : : * * * : : . . . . . : . . . . .	
<i>A. kurodai</i>	ERRDNL TGELRFE PMSDIDDIKSS IHAQDVS DTLAA INKATS GR LTVTMYNYAS-FTPLKSWNVD-YSMN-TTADSVY TASVSQIV	809
<i>C. elegans</i>	LHNI TVN-----VQMLSWGNGLD PII L TNEFHI DSV PAGSSEV LKGTGI TFSKI	782
<i>H. sapiens</i>	HSDY SMT-----LSVRVHTWSS-LE PVC SRV TERFVMKGG EAVCLYEEP VSE LL	758
<i>A. aculeatus</i>	AAGSVLTWR-----DLSGKPIAS -NGGLPTKPLPF HVGALNSTR LYRMMNKQQL	810
	: . . . . . : . . . . . : . . . . . : . . . . . : . . . . .	
<i>A. kurodai</i>	SEANCVSRDNCFLHFS TADKSNVST S WFLFSY PKYSN-LPRAQI KVVAVRNIS PRVLEVDIS SDAFAL YWVLSAG-DVLGRFSDN	894
<i>C. elegans</i>	TELS EYL YVS TLYDSS GVKIHE DVL VDFLEVD FNTFG---DVQISDVQRIDEKT YDLTIT TDRVSPFTWITC KKFPTGWFSDN	864
<i>H. sapiens</i>	RRCGNCTRES CVV SFYLSADHE LLSPTNYHFLSS PKEAVGLCKAQI TAIISQ--QGDI FVFDLE TSAVAPFVWLDVG-SIPGRFSDN	842
<i>A. aculeatus</i>	PRHEDAI LALELTATGSLPNTDEEV TTFHEQWFT PAF PKDLDL VNL RVRVEYDAP LKGF AVEATAG-VAL YTWLEH PEGVVG YFEEN	896
	. . . . . : : . . . . . : . . . . . : . . . . . : . . . . . : * * . . .	
<i>A. kurodai</i>	GFHLHTPTATVK-FYAADDVTA AKLQGN LKVKSL SDVQ---	931
<i>C. elegans</i>	GFHMIQR LRKIR-LIAKFEVDLE--KSDFTV CNLKNCYV--	900
<i>H. sapiens</i>	GFLMTEKTRTIL-FYPWEP TSKNELEQS FHV TSLTDIY---	879
<i>A. aculeatus</i>	SFVVVPGQKKVVG FVVQADETDGEVVDVTVRSLWDLNEGE	937
	. * : . . : : . . * . * :	