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Citation	Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 162(1-3), 24-33 https://doi.org/10.1016/j.cbpb.2012.02.003
Issue Date	2012-05
Doc URL	https://hdl.handle.net/2115/49356
Type	journal article
File Information	CBPB162-1-3_24-33.pdf

Characterization of a β-D-mannosidase from a marine gastropod, *Aplysia kurodai*

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Abstract

A β-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 Km and Vmax values for AkMnsd determined at pH 6.0 and 30°C with *p*-nitrophenyl β-D-mannopyranoside were 0.10 mM and 3.75 μmol/min/mg, respectively. AkMnsd degraded various polymer mannans as well as mannooligosaccharides 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 β-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) β-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; β-mannosidase; β-1,4-mannan; mannooligosaccharides.

1. Introduction

β-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 β-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 β-1,4-mannanase (EC 3.2.1.78, term β-mannanase in the present study) that degrades polymer mannans in an endolytic manner. Another is the β-D-mannosidase (EC 3.2.1.25, term β-mannosidase in the present study) that degrades mannan-oligosaccharides to mannose units. Since β-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, β-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, β-mannosidase is useful for

saccharification of mannans from decayed plant materials in a nutritional purpose for farm animals. β-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 β-mannosidase is important for releasing of mannose residues of N-glycosyl proteins in lysosome, defect of lysosomal β-mannosidase causes accumulation of mannosidic intermediates in cytosol and gives rise to the relating diseases (Neufeld, 1991). Thus, β-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., β-mannanases from plants, bacteria, fungi, and invertebrates, not so much attention has been paid to β-mannosidase. To date, characterization and primary structure analysis of β-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 β-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 β-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 β-mannosidase. Thus, the GHF to which the molluscan β-mannosidases belongs has not been revealed.

Recently, we isolated and characterized AkMan, a β-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 β-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 β-1,4-mannosyl linkages of the seaweeds' β-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 β-mannosidase.

Therefore, in the present study, we attempted to isolate the β-mannosidase from *A. kurodai*. As a result, a β-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 β-mannosidase from invertebrates.

2. Materials and methods

2.1. Materials

A synthetic substrate, *p*-nitrophenyl β-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, Bucking Ham shire, 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 β-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 DH5α 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 β-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. β-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 β-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 β-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 β-mannosidase activity

β-Mannosidase activity was assayed at $30^{\circ}C$ in a 1 mL of reaction medium containing 2.5 mM *p*-nitrophenyl β-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_2CO_3 to the reaction medium and the *p*-nitrophenol produced was determined by measuring the absorbance at 410 nm. One unit of β-mannosidase activity was defined as the amount of enzyme catalyzing the release of 1.0 μmol 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 β-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 (Km) and the maximal reaction velocity (Vmax), 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 β-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 mannooligosaccharides

Degradation products of mannan and mannooligosaccharides produced by AkMnsd were analyzed by thin-layer chromatography (TLC) as follows. Linear mannan from *C. fragile* and mannooligosaccharides (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 µL of each substrate solution, 25 µL of β-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 µL of reaction mixture at 30°C and at an appropriate time interval 10 µL of samples were withdrawn from the reaction mixture. The samples were then heated at 100°C for 2 min to terminate the reaction. Two micro litters 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 β-mannosidase (AkMnsd) and β-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 µL (0.25 unit) of each enzyme was incubated at 30°C for 0-24 h. An aliquot (10 µL) of the reaction mixture was withdrawn at appropriate time intervals and heated at 100°C for 2 min to inactive 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 ~1 mm^3, destained and dehydrated with solution (50% acetonitrile in 25 mM NH_4HCO_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 NH_4HCO_3), washed with 25 mM NH_4HCO_3 and alkylated with 55 mM ICH_2CONH_2 in 25 mM NH_4HCO_3. The gel pieces were dehydrated again with 50% acetonitrile in 25 mM NH_4HCO_3 and dried up completely in a vacuum desiccator. The protein in the gel pieces was then digested with 0.1 μg/mL trypsin in 25 mM NH_4HCO_3 at 37°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°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 μL 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 $MgCl_2$, and 5 pmol/μL primers, 1 ng/μL template DNA, and 0.5 units AmpliTaq Gold DNA polymerase. A successive reaction at $94^{o}C$ for 30 s, $55^{o}C$ for 30 s, and $72^{o}C$ 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* DH5α. The transformants were grown in 2 × YT medium supplemented by 50 μg/mL ampicillin at $37^{o}C$ 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, Km and Vmax, of AkMnsd to the substrate p-nitrophenyl β-D-mannopyranoside were estimated to be 0.10 mM and 3.75 U/mg, respectively.

3.2. Degradation of mannooligosaccharides and linear mannan by AkMnsd

To examine the activity of AkMnsd toward oligosaccharide substrates, mannooligosaccharides (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 mannooligosaccharides 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 β-mannan substrate (Fig. 3C). Thus, AkMnsd produced only mannose from the linear β-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 β-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 YERVPLDGQLNWMLSEASAGVNIPASVPGSMYTALLEKNL by the protein sequencer. This sequence showed 22.5% amino-acid identity with the GHF2 β-mannosidases from *Homo sapiens* (Alkhayat 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 β-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 β-

mannosidases belonging to glycoside hydrolase family 2 (GHF2) (Fig. 6). The amino-acid sequence of AkMnsd showed 43%, 24% and 20% identities with β-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 β-1,4-mannanase from *A. kurodai* (Zahura et al., 2010), we noticed the occurrence of β-mannosidase(s) in the crude enzyme preparation. In the present study, we successfully purified the β-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 β-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); *Tricoderma reesei*, 105±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 β-mannosidases.

The optimum pH and temperature of AkMnsd was pH 4.5 and 40°C, respectively (Fig. 2A and 2C). A β-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 β-mannosidase from a terrestrial mollusk, *H. pomatia,* were at pH 4.0 and 55°C (McCleary, 1983). Most of fungal β-mannosidases

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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). β-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 β-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 Km and Vmax values for AkMnsd were 0.10 mM and 3.75 U/mg, respectively. More or less similar Km and Vmax values were found in β-mannosidases from *T. fusca* TM51 (Km=180 μM and Vmax=5.96; Beki et al., 2003) and *T. reesei* (Km=0.12 mM; Kulminskaya et al., 1999). On the other hand, significantly higher Km and Vmax values were determined for the β-mannosidase from *A. fasciata*, i.e., 2.4 mM and 50.3 μmol/min/ mg, respectively (Andreotti et al., 2005). Significantly higher Km values i.e., 1.43 mM and 6.5 mM were determined for other molluscan β-mannosidase from *H. pomatia* (McCleary, 1983) and *A. fulica* (Sugahara et al., 1972), respectively. The Km values for the β-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 Km 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 mannooligosaccharides (M2~M6) (Fig. 3A). This indicated that AkMnsd is a typical β-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 β-1,4-mannosidic

bond of mannooligosaccharides. Such an exotype 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 mannooligosaccharides 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 β-mannosidases from animal and fungus sources (Alkhayat et al., 1998; Takada et al., 1999). GHF2 includes various types of polysaccharide-degrading enzymes such as β-galactosidase,

β-glucuronidase, exo-β-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 β-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 β-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 β-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 β-mannosidase.

Acknowledgment

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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 β-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 $30^{o}C$ in reaction mixtures containing 2.5 mM *p*-nitrophenyl β-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 $40^{o}C$ for 20 min in 50 mM sodium phosphate buffer (pH 2.0-9.0). The remaining activity was assayed at $30^{o}C$ in a medium containing 50 mM sodium phosphate buffer pH 6.0 and 2.5 mM *p*-nitrophenyl β-D-mannopyranoside. (C) Temperature dependence of AkMnsd. Activity was assayed at $15\text{-}60^{o}C$ in a reaction mixture containing 2.5 mM *p*-nitrophenyl β-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 mannooligosaccharides and linear mannan produced by AkMnsd. (A) Degradation of mannose (M1) and mannooligosaccharides (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 µL of purified enzyme (0.25 unit) was mixed and allowed to react at 30°C for 0-24 h and 2 µL 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 µL (0.25 unit) of each enzyme was allowed to react at 30°C for 0-24 h and 2 µL 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 β-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^a/mg)	Yield (%)	Purification (fold)
Crude[b]	730.36	795.66	1.09	100	1
Butyl[c]	23.08	205.76	8.92	25.86	8.18
DEAE[d]	12.79	142.85	11.17	17.95	10.24
Gelfiltration[e]	2.21	48.15	21.80	6.05	20.00
Gelfiltration[f]	0.85	21.71	25.79	2.72	23.66

[a] One unit of β-mannosidase activity was defined as the amount of enzyme that releases of 1.0 μmol of *p*-nitrophenol per minute from 2.5 mM *p*-nitrophenyl β-D-mannopyranoside.

[b] Crude enzyme after the dialysis against 2 mM sodium phosphate buffer (pH 6.0).

[c] Active fractions obtained by TOYOPEARL Butyl-650 M chromatography.

[d] Active fractions obtained by TOYOPEARL DEAE-650 M chromatography.

[e] Active fractions obtained by the first gel filtration through Superdex 200 10/300 GL.

[f] 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[a]	Sequence
N-terminal	YERVPLDGQLNWMLSEASAGVNIPASVPGSMYTALLEKNL
T-1	EGDNSIVVGFR
T-2	INGLPVFLKGSNWIPADNFQER
T-3	TDYIYLTQINQAMSM
T-4	HAQDVSDTLAAINK
T-5	TSTSWLFLSYPK

[a] T-1–T-5, tryptic fragments.

Table 3. Primers used for amplification of AkMnsd-cDNA

	Primer names	Sequences[a, b]
AkMnsd-cDNA-1	AkMnsdFw	5'-GARGCNTTYGCNGGNGTNAAYATHCC-3'
		(EASAGVNIP)
	AkMnsdRv	5'-CATNSWCATNGCYTGRTTDATYTGNGT-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′

[a] 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.

[b] Amino-acid sequences used for designing the degenerated primers are in the parentheses.

Fig. 1

Fig. 2

Fig. 3

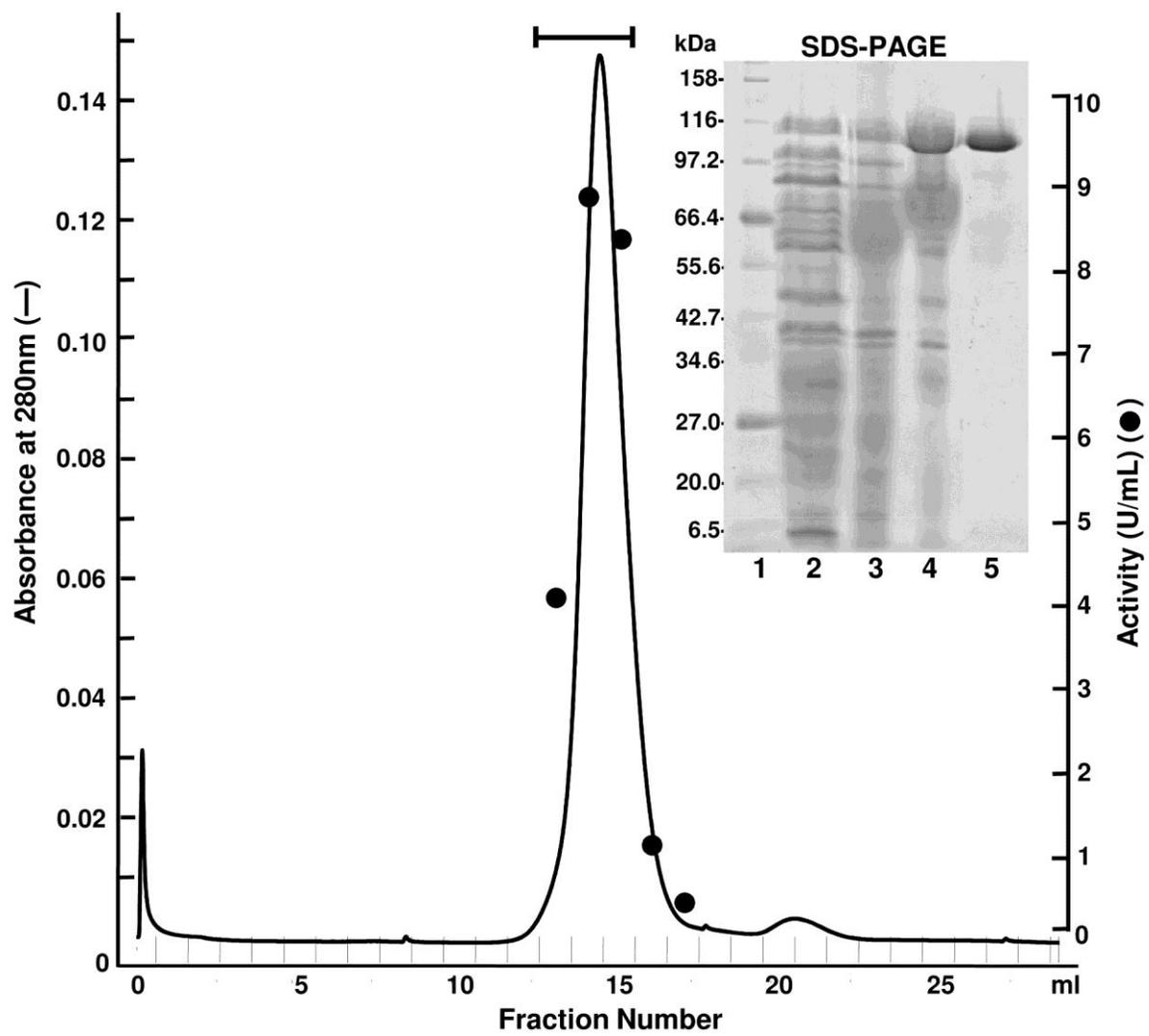**Fig. 4**

(A)

M1-
M2-
M3-
M4-
M5-
M6-

Mk 0   30 | 1   2   4   6   12   24 |
     min   h
        Reaction time

(B)

M1-
M2-
M3-
M4-
M5-
M6-

-tri
-pen
-hex

Mk 0   30 | 1   2   4   6   12   24 |
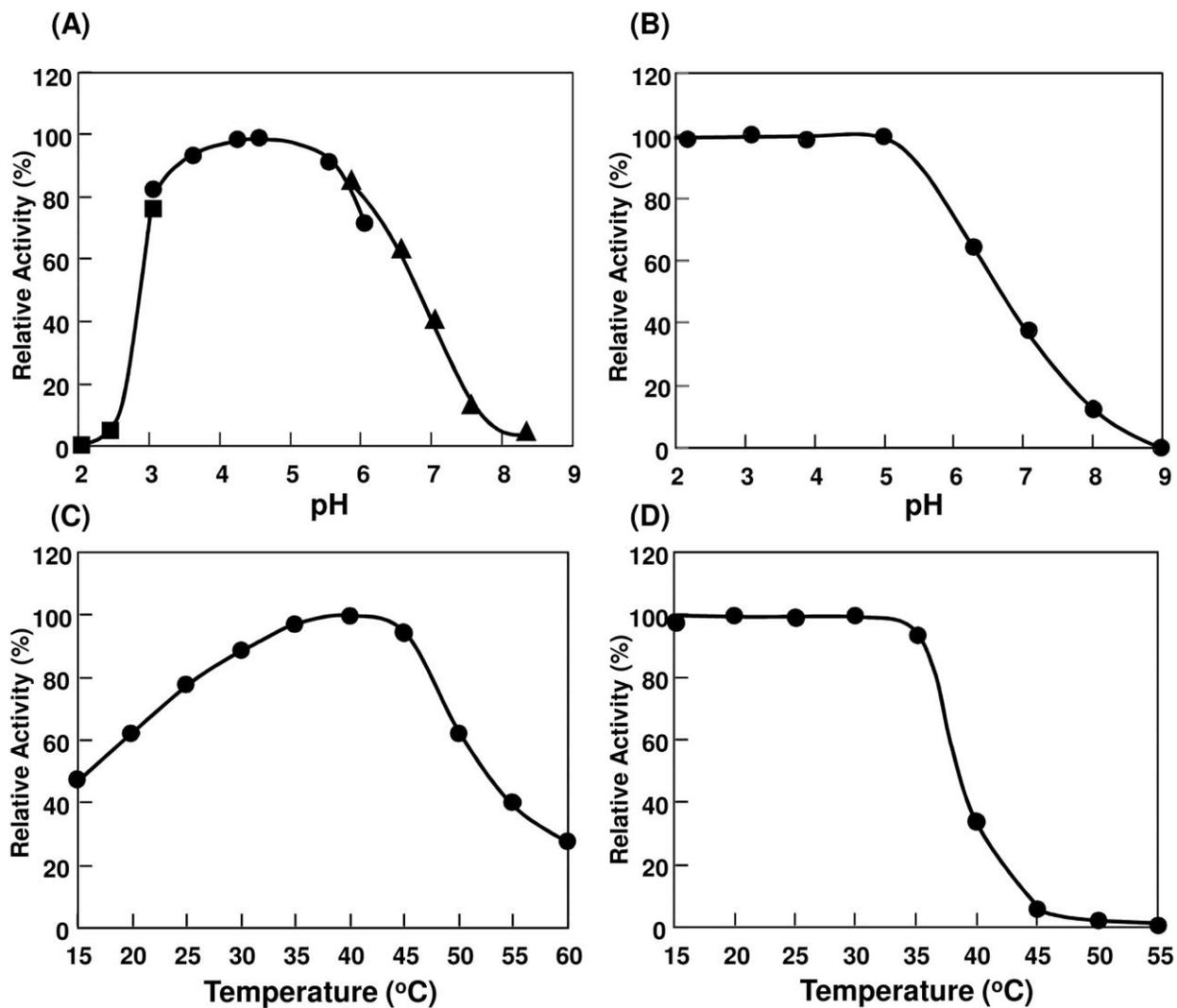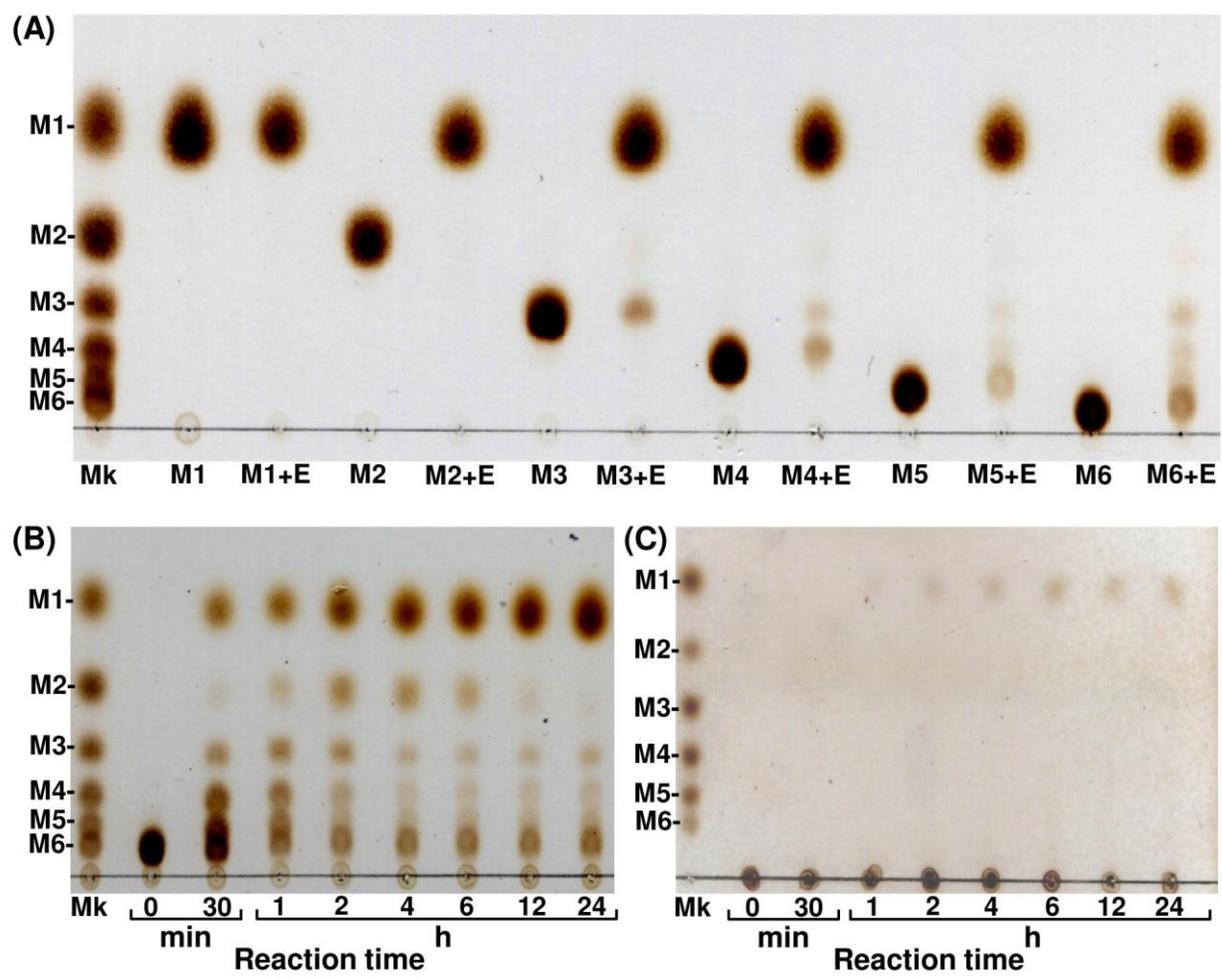   min   h
        Reaction time

34
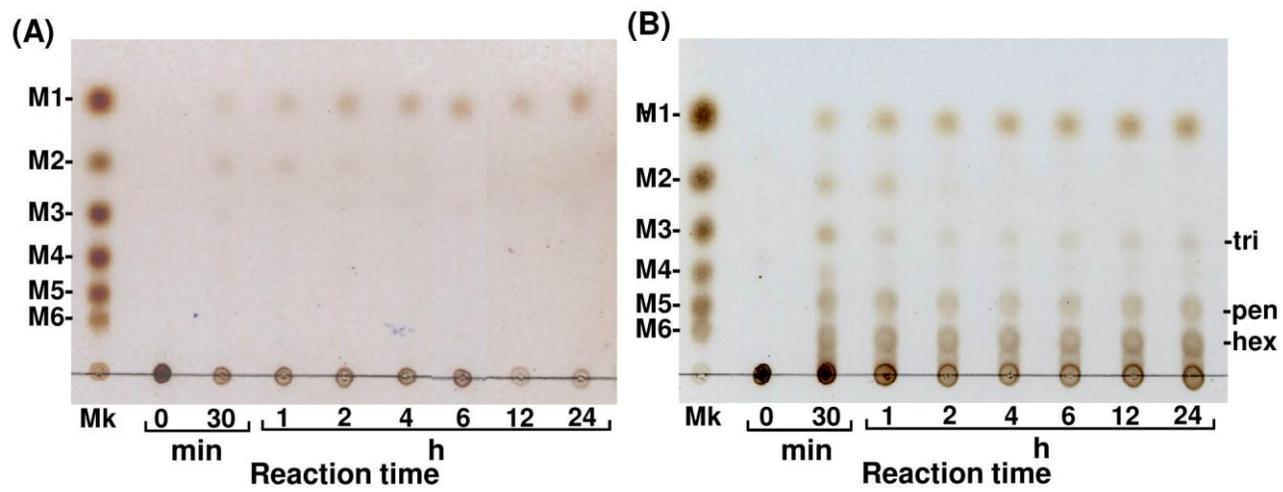
Fig. 5

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AGAAGTCCAGCTACGAGATCCCGCCCAACTGTCCACCCCCGGTCCAGCAAGGCAGTTCACAAGTGTCGCTGGCAGCAGAAGAACCACAGACTGCGGCATA    100
GGCAAGATGTTTTGGTTCCACGTCTCGCTTCTGATTGCTACTGGCATTTCACAGTGGATGCTCAAAACGAATAATTTGGTCAGCTCTTATGAACGGGTTC    200
      M  F  W  F  H  V  S  L  L  I  A  T  G  I  S  Q  W  M  L  K  T  N  N  L  V  S  S  Y  E  R  V     31
                        Signal peptide
CTCTCGATGGGCCAGCTTAATTGGATGTTGTCAGAGGCATCTGCAGGTGTGAATATACCGGCGTCTGTCCCCGGATCCATGTACACGGCTCTGTTGGAAAA    300
P  L  D  G  Q  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
GAACCTCATCCAAGACCCTCTCTATCGGGACAACGACAACAAATATGCCTGGTTGGGTAACTCGGACTGGACGTACAACAGGAACTTCACAGTGTCCAAA    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
TCTGTGGCTGAGTCCAGCAAGGTGCTGCTGGTCTGTGAGGGGCTGGACACAGTGGCGTCAGTCGTGGTCAACGGAAAGGCAGTGGGCGACACAGACAACA    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
TGTTTGTCCGCCATGCCTTTGACGTCACCGGGATCGTGAAGGAAGGTGACAACAGTATTGTCGTAGGCTTCCGGTCAGCGGTGCTTGAGGCACAGGAGCG    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  E  R     165
                                                         T-1
CGACCAGAAGTCCAGCTACGAGATCCCGCCCAACTGTCCACCCCCGGTCCAGCAAGGCCAGTGTCACGTGAACCAGCTGAGGAAGGAGCAGTGTAGCTTC    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
AGCTGGGACTGGGGACCCTCCTTCCCCACACAGGGCATCTGGAAGCCAATCTATCTGAATGCGTTTAGTACCGCCGTAGTGCAACGAGTCTCCGCTATAC    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
CTAAGAAAGTGAGTGGACAGTGGCAGGTGGACGTGGATGTAATCTTTGATGTCAACGAGGGTTTAACAGAGGTCAAGGGTCAAGTGCAAGTGGACGTACC    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
GGCCTTGAACTTGACCTTGGTTCAGGACGTGACAGTTTCTTCCTCCCAGACAGGGCTAAGGTCGTACTCAAAGTGCCGCTGGCCAGGGATGTGCTGCTG    1000
 A  L  N  L  T  L  V  Q  D  V  T  V  S  S  S  Q  S  R  A  K  V  V  L  K  V  P  L  A  R  D  V  L  L     298
TGGTGGCCAAACGGTTACGGTCTCCAGAACCTGTACCAGATGTCCGTGACCTTCACCTCTGGCTCTGACGTCTCCAGCCGCTCGTTCAAGGTTGGCTTCA    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
GGACAGTGGAGCTGATCCAAGACCCCGTGTCTTCCGACTCTAAGAAAGGCCTGACCTTCTACTTCCGCATCAACGGTCTCCCGGTGTTCCTGAAAGGCTC    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
                                                          T-2
CAACTGGATTCCGCCGACAACTTCCAGGAGCGCATCACCAGAGACAGACTGAGCTACCTGCTGCAGTCGACGGCCACTGTGGGCATTAACTCCATGCGC    1300
 N  W  I  P  A  D  N  F  Q  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
     N-WIPADNFQER    T-2
GTGTGGGGCGGTGGGGTGTACGAGTCTGAAGATTTCTACGAACTGTGTGATGAGTTTGGTATCATGGTGTGGCAAGACCTCATGTTCAGTGTCGCTCTTT    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
ACCCGACCTACGATGGCTTCCTCCAGTCGGTGGCCACAGAGATCAGACAACAGGTCCAACGCCTCAAACACCACCCGTCCATCATTCTGTGGGCTGGCAA    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
CAACGAGAACGAGAAAGGTCTGCGCCAGAACTGGTTCAAAACACAGAACAACTTCACGCTCTATTACAACGATTATGTGAAGCTCTACGTCACAACCATC    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
AAACCCATAGTAAACGCCGAGGACGACAGCCGTGAGTACCTTCTCTCCAGTCCTTCCAACGGCAAAGAGAGTGAGAAAGAGGGTTATGTAGCCAAGGAGC    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
CTTGGAGTGAACTCTATGGAGACATTCATTTCTACGAAAACGTCGTGGATCAGTGGAACCCGGCCGTGTTCCGCGTGCCCCGCATGGCCTCAGAGTACGG    1800
 P  W  S  E  L  Y  G  D  I  H  F  Y  E  N  V  V  D  Q  W  N  P  A  V  F  R  V  P  R  M  A  S  E  Y  G     565
CATACAGGGCTGGTGCAACAACGAGAGTCTGGCCTCAGTATTCGCCCCAGAGGACTACAGCATGGACGCGCCCATGATAGGACACAGGCAGCATCATTAT    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
AACGGCAACGCTGAAATGGCAGCAGAAGTGGCTCTGCACATAGACTTGTCCACTTCATCGGATCCTGTCGTCAAGTTCACCGACTACATCTACCTTACAC    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
                                                                        T-3
AGATCAACCAGGCCATGTCCATGCGTACCCAGAGCGAGCACTACAGGCGCTTCCAGTCCCGACTGCTCTCTGATGGCCGCGGACTCACCATGGGCGCCCT    2100
Q  I  N  Q  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
CTACTGGCAGCTCAACGACATCTGGCAGGCTCCCACCTGGGCCTCCATTGACTATGAGGGCCGCTGGAAGATGTTGCATTACTACGCCCAGCACTTCTTC    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
CAGCCCACCCTGGTGTCCCCGTACCTGCAGGACAACAACACCCTCAACGTCTTCATGGCCGTGGATGGGGTCCCCAGTCTGGAGCGACGCGACAATTTGA    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
CAGGTGAACTGAGATTTGAGCCCATGAGCGATATTGACGACATCAAGAAGAGCAGTATACACGCACAAGACGTCTCCGACACTTTGGCAGCCATTAACAA    2400
T  G  E  L  R  F  E  P  M  S  D  I  D  D  I  K  K  S  S  I  H  A  Q  D  V  S  D  T  L  A  A  I  N  K     765
                                                       T-4
AGCAACTTCCGGTCGTTTGACGGTGACCATGTACAATTACGCCAGTTTCACCCCCCTCAAGTCCTGGAATGTTGATTATTCCGATGAACAACAACGGCCGAC    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
TCTGTGTACACGGCCAGTGTGAGCCAGCTCGTGAGCGAGGCCAACTGTGTGTCCAGGGACAACTGTTTCTTACACTTCTCCACAGCAGACAAAAGTAACG    2600
 S  V  Y  T  A  S  V  S  Q  L  V  S  E  A  N  C  V  S  R  D  N  C  F  L  H  F  S  T  A  D  K  S  N     831
CCGTGACCTCCACCTCTTGGCTTTTCCTCTCTTACCCCAAGTATTCCAACTTGCCTCGAGCTCAAATCAAGGTGGTCGCAGTCAGGAACATCTCTCCACG    2700
A  V  T  S  T  S  W  L  F  L  S  Y  P  K  Y  S  N  L  P  R  A  Q  I  K  V  V  A  V  R  N  I  S  P  R     865
       T-5
AGTTTTGGAAGTGGACATCTCCTCTGATGCTTTTGCTCTTGTACGTCTGGCTATCTGCTGGGGACGTCCTGGGCAGGTTCTCGGATAACGGCTTTCACCTT    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
CACACCCCCGACAGCCACGGTCAAGTTCTATGCCGCGGACGATGTGACGGCTGCCAAGCTGCAGGGAAATCTCAAAGTGAAAAGTTTGTCCGATGTCCAA    2899
 H  T  P  T  A  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
TAGGAAGATGATAATAGAGTGGAGTTCGTTTGAACCGAACACGGTTGATCCGAACACAGCCGGTAAAATGAAAGAGAAAAAAAAAAAA                 2985
 *
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Fig. 6

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A.kurodai    MFWFHVSLLIATGISQWMLKTNNLVSSYERV--PLDGQLNWMLSEASAGVNIPASVPGSMYTALLEKNLIQDPLYRDNDNKYAWLGN   85
C.elegans     MRTSLVVCLFWLLFQLHTTHGYNTLVNLAG-----NWEFSSSNKTVNGTGTVPGDIYSDLYASGIIDNPLFGENHLNLKWIAE      78
H.sapiens     MRLHLLLLLALCG----AGTTAAELSYS-----LRG--NWSICNGNGSLELPGAVPGCVHSALFQQGLIQDSYYRFNDLNHRWVSL    75
A.aculeatus   MRALPTTATTLLGVLFFPSASRSQYVRDLG----TEQWTLSSATLNRTVPAQFPSQVHMDLLREGIIDEPYNDLNDFNLRWIAD      80
                          ::      ..          :* :....   ..*.::  *  ..:*::.    *.:  *:.

A.kurodai    SDWTYNRNFTVSKSVAESSKVLLVCEGLDTVASVVVNGKAVGDTDNMFVRHAFDVTGIVKEGDNSIVVGFRSAVLEAQERDQKSS--  170
C.elegans    DDWTYSRKFRL-IDLDDTVGAFLEIESVDTIATVYVNGQKVLHSRNQFLPYHVNVTDIIALGENDITIKFKSSVKYAEKRADEYKKI  164
H.sapiens    DNWTYSKEFKIPFEISKWQKVNLILEGVDTVSKILFNEVTIGETDNMFNRYSFDITNVVRD-VNSIELRFQSAVLYAAQQSKAHTX-  160
A.aculeatus  ANWTYTSGKIEGLGEDYES-TWLVFDGLDTFASISFCGQFVGATDNQFRQYMFDVSSILKACPEEPTLGIQFGSAPNIVDAIAQDP-  165
              :***.             . *  :.::**.:.:.    :   : * *  : .::::.::    :.  : ::  .

A.kurodai    --YEIPPNCPPPVQQGQCHVN--QLRKEQCSFSWDWGPSFPTQGIWKP-----------IYLNAFSTAVVQR--VSAIPKKVSGQWQ  240
C.elegans    FGHSLPPDCNPDIYHGECHQN--FIRKAQYSFAWDWGPSFPTVGIPST-----------ITINIYRGQYFHDFNWKTRFAHGKWKVA  238
H.sapiens    --YQVPPDCPPLVQKGECHVN--FVRKEQCSFSWDWGPSFPTQGIWKD----------VRIEAYNICHLNYFTFSPIYDKSAQEWN   232
A.aculeatus  ----SSPTWPEGVQITYEYPNRWFMRKEQSDFGWDWGPAFAPAGPWKPGYVVQLKQAAPVYVRNTDLDIYRLGQINYLPPDQTQPWV  248
                   .*      :  :  * :  :** *.*.*****:**.. *  .           ::.       ::    .  .

A.kurodai    VDVDVIFDVNEGLTEVKGQVQVDVPALNLTLVQDVTVSSSQSRAKVVLKVPLARDVLLWWPNGYGLQNLYQMSVTFTSGSDVS--SR  325
C.elegans    FEFDTFHYGARTIEYSVQIPELGIKESDYYRLSATKSLQTRSKNIMSLSIPMEHEPERWWPNGMGEQKLYDVVVSMGGQVKEK----  321
H.sapiens    LEIESTFDVVS-SKPVGGQVIXAIPKLQTQQTYSIELQPGKRIVELFVNISKNITVETWWPHGHGNQTGYNMTVLFELDGGLNI-EK  317
A.aculeatus  VNASLDYLGSLPENPSMAIEVKDLQSGEILASRPLTNITVTEGSVTGVTVLEGVDPKLWWPQGLGDQNLYNVTISVTDGGNQSVAEV  335
              .:.  .        :   :         :  :           :.:        ***:* * *. *:: :.       .

A.kurodai    SFKVGFRTVELIQDPVSSDSK-K----GLTFYFRINGLPVFLKGSNWIPADNFQ-ERITRDRLSYLLQSTATVGINSMRVWGGGVYE  406
C.elegans    --KIGFKTVELVQDLIDPKKPEK----GRNFYFKINDEPVFLKGSNWIPVSMFRSDRENIAKTEFLLDSVAEVGMNAIRVWGGGFYE  402
H.sapiens    SAKVYFRTVELIEEPIKGSP-------GLSFYFKINGFPIFLKGSNWIPADSFQ-DRVTSELLRLLLQSVVDANMNTLRVWGGGIYE  396
A.aculeatus  TKRTGFRTIFLNQRNITDAQLAQGIAPGANWHFEVNGHEFYAKGSNLIPPDCFW-TRVTEDTMTRLFDAVVAGNQNMLRVWGGSGAYL  421
               :  *:*:* *:  :        * .::*.:*.  .: **:* ** . *   *     *:::..  . * :***..** 

A.kurodai    SEDFYELCDEFGIMVWQDLMFSVALYPTYDGFLQSVATEIRQQVQRLKHHPSIILWAGN[N]ENEKGLRQNWFKTQN-NFTLYYNDYVK  492
C.elegans    SNHFYYYASKKGILVWQDLMFACALYPTTEEFIQNAEEEVSYNVDRISQHTSVIVFSGN[N]ENEAAIRGHWWKASNYTESQQVKDYVL  489
H.sapiens    QDEFYELCDELGIMVWQDFMFACALYPTDQGFLDSVTAEVAYQIKRLKSHPSIIIWSGN[N]ENEEALMMNWYHISFTDRPIYIKDYVT  483
A.aculeatus  HDYIYDLADEKGILLCSEFQFSDALYPTDDAFLENVAAEVVYNVRRVNHHPSLALWAGG[N]EIESLMLLLVEAADPESYPFYVGEYEK  508
              : :*  ..: **:: .:: *: ***** : *:.   *:  :: *:. *.*:::*.** *     :         .       ::

A.kurodai    LYVTTIKPIVNAEDDSREYLLSSPSNG-------KESEKEGYVAKEPWSELYGDIHFYEN--VVDQWNPAVFRVPRMAS[E]YGIQGWCN  571
C.elegans    LYQR-LAKIAKKVAPTIPFIMSSPSNG------VETEEEGGVSKNPYDVRYGDIHYYNE-FVNLWRDDTYLTPRCAS[E]YGVQSYPM   567
H.sapiens    LYVKNIRELVLAGDKSRPFITSSPTNG-------AETVAEAWVSQNPNSNYFGDVHFYDY-ISDCWNWKVFPKARFAS[E]YGYQSWPS  562
A.aculeatus  MYISLFLPLVYENTRSISYSPSSTTEGYLDIDLSAPVPMAERYSNTTEGEYYGDTDHYNYDASIAFDYGTYPVGRFAN[E]FGFHSMPS  595
              :*   :  :.    :   : **.::*             ::  .  :** ..:       :  .:    * .*.*:* :.

A.kurodai    NESLASVFAP-EDYSMDAPMIGHRQHHYNGNAEMAAEVALHIDLSTSS-------------DPVVKFTDYIYLTQINQAMSMRTQS  643
C.elegans    KETMLNWINE-SDWEYTSKAMFHRQHHPGGIATNLLMIFQHLPIPAECGSKSVSDVPSCKYISSASYMSRLAYFSQVHQSIALKTQT  653
H.sapiens    FSTLEKVSST-EDWSFNSKFSLHRQHHEGGNKQMLYQAGLHFKLPQST-------------DPLRTFKDTIYLTQVMQAQCVKTET  634
A.aculeatus  LQTWQQALTDPADLTFNSSVVMLRNHHYPAGGLMTDNYHNTVARHGRNDPGR---------AGLLPDAQHSVRPRGQLQRLVPRDPA  673
                .:   .     .:    *.**:.:*                    *         *      :.

A.kurodai    EHYRRFQSRLLSDGRGLTMGALYWQLNDIWQAPTWASIDYEGR-----WKMLHYYAQHFFQPTLVSPYLQDNNTLNVFMAVDGVPSL  725
C.elegans    LHYRRFRNTTTNEGLGNTMCAMYWQLNDVWAAPTWSTIDFEQN-----WKMAHYEARRFFSNVAVYSFADETDFNLKVFLLNDNPYL  735
H.sapiens    EFYRRSRSEIVDQ-QGHTMGALYWQLNDIWQAPSWASLEYGGK-----WKMLHYFAQNFFAPLLPVGFENEN-----TFYIYGVSDL  710
A.aculeatus  LPGGPLQVTNPVLPAGQRAARTPARVPVLAARGHLAGALVGGDRVRRPLEGPHYVARDIYKPVIVSPFWNYTTGALDIYVTSDLWTA  760
               :       .    *             ::        :            :  ** *: ::        ::.        .

A.kurodai    ERRDNLTGELRFEPMSDIDDIKKSSIHAQDVSDTLAAINKATSGRLTVTMYNYAS-FTPLKSWNVD-YSMN-TTADSVYTASVSQLV  809
C.elegans    LHNITVN--------------------------------------VQMLSWGNGLDPILTNEFHIDSVPAGSSEVLKTGITFSKI    782
H.sapiens    HSDYSMT--------------------------------------LSVRVHTWSS-LEPVCSRVTERFVMKGGEAVCLYEEPVSELL  758
A.aculeatus  AAGSVTLTWR------------------------------------DLSGKPIAS-NGGLPTKPLPFHVGALNSTRLYRMNMKQQPL  810
                                                            .  ..        :    .

A.kurodai    SEANCVSRDNCFLHFSTADKSNAVTSTSWLFLSYPKYSN-LPRAQIKVVAVRNISPRVLEVDISSDAFALYVWLSAG-DVLGRFSDN  894
C.elegans    TELSEYLYVSTLYDSSGVKIHEDVLVPDFLFEVDFNTFG-----DVQISDVQRIDEKTYDLTITTDRVSPFTWITCKKPFTGWFSDN  864
H.sapiens    RRCGNCTRESCVVSFYLSADHELLSPTNYHFLSSPKEAVGLCKAQITAIISQ--QGDIFVFDLETSAVAPFVWLDVG-SIPGRFSDN  842
A.aculeatus  PRHEDAILALELTATGSLPNTDEEVTFTHEQWFTPAFPKDLDLVNLRVRVEYDAPLGKFAVEATAG-VALYTWLEHPEGVVGYFEEN  896
                .       .            :             ::                 .  :..: :.*:    .  * * *.:*

A.kurodai    GFHLHTPTATVK-FYAADDVTAAKLQGNLKVKSLSDVQ---  931
C.elegans    GFHMIQRLRKIR-LIAKFEVDLE--KSDFTVCNLKNCYV-- 900
H.sapiens    GFLMTEKTRTIL-FYPWEPTSKNELEQSFHVTSLTDIY--- 879
A.aculeatus  SFVVVPGQKKVVGFVVQADETDGEWVHDVTVRSLWDLNEGE 937
             .*:    .: :        .. * .*.:
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