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Comprehensive enzymatic analysis of the amylolytic system in the digestive fluid of the sea hare, *Aplysia kurodai*: Unique properties of two α-amylases and two α-glucosidases

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ABSTRACT

Sea lettuce (*Ulva pertusa*) is a nuisance species of green algae that is found all over the world. East-Asian species of the marine gastropod, the sea hare *Aplysia kurodai*, shows a clear feeding preference for sea lettuce. Compared with cellulose, sea lettuce contains a higher amount of starch as a storage polysaccharide. However, the entire amylolytic system in the digestive fluid of *A. kurodai* has not been studied in detail. We purified α-amylases and α-glucosidases from the digestive fluid of *A. kurodai* and investigated the synergistic action of these enzymes on sea lettuce. *A. kurodai* contain two α-amylases (59 and 80 kDa) and two α-glucosidases (74 and 86 kDa). The 59-kDa α-amylase, but not the 80-kDa α-amylase, was markedly activated by Ca^{2+} or Cl^{-}. Both α-amylases degraded starch and maltoheptaose, producing maltotriose, maltose, and glucose. Glucose production from starch was higher with 80-kDa α-amylase than with 59-kDa α-amylase. Kinetic analysis indicated that 74-kDa α-glucosidase prefers short α-1,4-linked oligosaccharide, whereas 86-kDa α-glucosidase prefers large α-1,6 and α-1,4-linked polysaccharides such as glycogen. When sea lettuce was used as a substrate, a 2-fold greater amount of glucose was released by treatment with 59-kDa α-amylase and 74-kDa α-glucosidase than by treatment with 45-kDa cellulase and 210-kDa β-glucosidase of *A. kurodai*. Unlike mammals, sea hares efficiently digest sea lettuce to glucose by a combination of two α-amylases and two α-glucosidases in the digestive fluids without membrane-bound maltase–glucoamylase and sucrase–isomaltase complexes.

1. Introduction

Marine algae can provide a high-yield source of biofuels without compromising food supplies, rainforests, or arable land [1,2]. Sea lettuce (*Ulva pertusa*) is a nuisance species of green algae that is found all over the world. Sea lettuce is consumed by sea animals. Particularly, East-Asian species of the marine gastropod, the sea hare *Aplysia kurodai*, shows clear feeding preference for sea lettuce. Therefore, research on the glucose production system of sea hares from seaweed polysaccharides could contribute important new insights into the development of biofuel processing technologies from seaweed. Recently, we purified four cellulases and two β-glucosidases from the digestive fluid of *A. kurodai*, investigated its entire enzymatic cellulolytic system [3], and showed unique properties of digestive enzymes that were not predicted from genetic approaches. The 45-kDa cellulase from *A. kurodai* possesses cellobiohydrolase and β-glucosidase activities in addition to β-1,4-endoglucanase activity. Multicatalytic activities including β-glucosidase, laminarinase and lactase activities, are possessed by the 210-kDa β-glucosidase.

During the course of purification of cellulolytic enzymes, amylase and α-glucosidase activities were also found in the digestive fluid of *A. kurodai,* suggesting that it utilizes starch in seaweed as an energy source in addition to cellulose. Sea lettuce, a staple food of sea hares, is an algae with high starch content [4]. The entire digestive amylolytic system of marine invertebrates feeding on seaweed or microalgae is poorly understood. The α-amylase family (glycoside hydrolase family 13, GHF13) is one of five structural families (GHF4, GHF13, GHF31, GHF97 and GHF122) of starch-

Abbreviations: ApAmy59, *Aplysia* 59-kDa α-amylase; ApAmy80, *Aplysia* 80-kDa α-amylase; ApAGL74, *Aplysia* 74-kDa α-glucosidase; ApAGL86, *Aplysia* 86-kDa α-glucosidase; CMC, carboxymethylcellulose; 4MU-α-Glu, 4-methylumbelliferyl α-glucoside; GHF, glycoside hydrolase family; PVDF, polyvinylidene fluoride; TLC, thin layer chromatography

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degrading hydrolases and includes specifically acting on the α-1,4- and α-1,6-*O*-glycosidic linkages of starch [5–7]. The members of GH13 are multidomain $(\beta/\alpha)_8$-barrel enzymes and display large variation on a structural theme providing an array of substrate specificity. α-Amylases [EC 3.2.1.1] and α-glucosidases [EC3.2.1.20] cloned from mollusks [8–10] and crustaceans [11,12] belong to GH13. In contrast to marine invertebrates, the digestive amylolytic system in mammals has been well characterized [13–16]. Salivary and pancreatic α-amylase [EC 3.2.1.1] catalyze random hydrolysis of α-1, 4 bonds and yield dextrin, a mixture of glucose, maltose, and maltotriose as major products. The maltase [EC 3.2.1.20]–glucoamylase [EC 3.2.1.3] and sucrase [EC 3.2.1.48]–isomaltase [EC 3.2.1.10] complexes located on the brush border membrane of intestinal mucosal cells catalyze hydrolysis of starch-derived products to glucose. The maltase–glucoamylase complex hydrolyzes far larger and more complex glucan structures, including amylopectin, glycogen, amylose, and α-limit dextrins, than does the sucrase–isomaltase complex. The α-1, 6 glycoside bond in limit dextrins is almost exclusively hydrolyzed by the isomaltase subunit of sucrase–isomaltase, thereby liberating nonreducing α-1, 6-linked glucose residues from dextrin.

To investigate the digestive amylolytic system in marine invertebrates, comprehensive enzymatic analysis of amylolytic glucanases in *A. kurodai* was performed. We purified two α-amylases (59 and 80 kDa) and two α-glucosidases (74 and 86 kDa) from the digestive fluid of *A. kurodai* at the mg level and analyzed their cleavage specificity, synergistic action, and glucose-producing activities from various seaweeds. Our findings provide the first example of an enzymatic process of glucose liberation from starch in the digestive fluid of invertebrates.

2. Materials and methods

2.1. Materials

A. kurodai (body length, 20–25 cm) and sea lettuce (*U. pertusa*) were collected on the coast of Naruto, Japan, during April–July. Sea hare and sea lettuce are not protected in this area. No specific permissions were required since collection of these species is allowed. The digestive fluid was obtained from the gastric lumen by squeezing the stomach after dissection and then fractionated using ammonium sulfate (0–60% saturation), as described previously [3]. The sea lettuce was washed with water, dried at 50 °C, and then minced in a Waring blender. Dried seaweeds (*U. prolifera*, *Saccharina* sp., and *Eisenia bicyclis*) and dried microalgae (*Chlorella vulgaris*) were purchased from a local grocery store and minced in a Waring blender.

Corn starch, D-(+)-glucose, and the Glucose CII Test Wako were purchased from Wako Pure Chemicals (Osaka, Japan). Carboxymethylcellulose (CMC; sodium salt, low viscosity), laminarin (β-1,3:1,6-glucan) from *Laminaria digitata*, and 4-methylumbelliferyl (4MU)-α-D-glucoside were from Sigma–Aldrich (St, Louis, MO). Maltoheptaose and hydroxyapatite were obtained from Seikagaku Kogyo (Tokyo, Japan). DEAE-Sepharose™ (fast flow), CM-Sepharose™ (fast flow), phenyl-Sepharose (HiLoad™ 16/10), Sephacryl S-100, Sephacryl S-200, and Mono-Q HR5/5 were obtained from GE Healthcare (Uppsala, Sweden). A peroxidase-labeled lectin kit was purchased from J-OIL MILLS (Tokyo, Japan). All other chemicals used were of analytical grade.

2.2. Enzyme assay

Amylase activity (endo-α−1,4-endoglucanase) was assayed in a 0.2-ml reaction mixture comprising 1% corn starch, 50 mM acetate buffer (pH 6.0), 10 mM $CaCl_2$ and an appropriate amount of enzyme at 37 °C. Following incubation at 3 °C for 10–30 min, the reaction mixture was terminated by heat treatment at 95 °C for 5 min. The quantities of reducing sugars liberated by the hydrolysis of corn starch were determined by the method of Nelson and Somogyi [17]. One unit (U) of enzyme activity was defined as that amount of enzyme that liberates reducing sugars equivalent to 1 μmol of glucose per min at 37 °C. α-Glucosidase activity was assayed using 4-methylmbelliferyl (4-MU)-α-glucoside, as described previously [3]. Released 4-methylumbelliferone was measured fluorometrically (excitation, 365 nm; emission, 450 nm). One unit (U) was defined as the activity that produced 1 μmol of 4-methylumbelliferone per min at 37 °C. Glucose liberated by hydrolysis of substrates was determined by the Glucose CII Test Wako kit using glucose oxidase. Protein concentration was determined by the Bradford method using BSA as the standard [18].

2.3. Purification of amylase and α-glucosidase from the digestive fluid

All purification procedures were performed at 4 °C. Amylase and α-glucosidase activities were measured by using corn starch and 4MU-α-glucoside, respectively, unless otherwise stated. A frozen ammonium sulfate fraction from 300 ml of the digestive fluid, prepared as mentioned above, was thawed and centrifuged at 12,000×*g* for 10 min. The supernatant was applied to a CM-Sepharose column (2.5 × 20 cm) equilibrated with 20 mM acetate (pH 6.0) and washed with the same buffer. Proteins bound to the CM-Sepharose column were eluted using a linear gradient of NaCl (0–0.3 M) in the same buffer as described previously [3]. The chromatography gave coelution of 59-kDa amylase and 45-kDa endo-β-1,4-glucanase. The fractions possessing the enzyme activity were concentrated by ultrafiltration, and ammonium sulfate was then added to produce the final concentration of 1 M. Following centrifugation, the supernatant was applied to a phenyl-Sepharose (HiLoad™ 16/10) column equilibrated with 20 mM Tris–HCl (pH 7.0) containing 1 M ammonium sulfate and washed with the same buffer. A linear gradient of ammonium sulfate (1–0 M) was used to elute bound proteins, and the eluant was fractionated into eight fractions (A–H), as described previously [3]. The purity of the enzyme was checked by SDS–PAGE using 10% acrylamide gel [19].

2.3.1. 74-kDa α-glucosidase (ApAGL74)

Components showing α-glucosidase activity in the CM-Sepharose unbound fraction were fractionated by DEAE-Sepharose chromatography, under conditions as described above. The α-glucosidase activity was detected in both DEAE-Sepharose unbound and bound fractions. The α-glucosidase in the DEAE-Sepharose bound fraction was further purified by phenyl-Sepharose chromatography, under conditions as described above. The fractions with α-glucosidase activity were applied to Sephacryl S-100 gel filtration and eluted with 20 mM Tris–HCl (pH 7.0) containing 0.1 M NaCl. The fractions with α-glucosidase activity were concentrated by ultrafiltration and dialyzed against 20 mM Tris–HCl (pH 7.5). The dialyzate was applied to a Mono Q HR5/5 column equilibrated with the same buffer and eluted with a linear gradient of NaCl (0–0.2 M) in the same buffer. The fractions with the activity were concentrated and subjected to a hydroxyapatite column (1.0 × 2.5 cm). The column was eluted with 20 mM Tris–HCl (pH 7.0) (A fraction) and followed by a linear gradient of sodium phosphate (0–0.2 M) (B fraction). α-Glucosidase activity was detected in both fractions. The A fraction contained a 74-kDa protein, and the B fraction contained 74-kDa and 190-kDa proteins. The A fraction was used as purified 74-kDa α-glucosidase.

2.3.2. 86-kDa α-glucosidase (ApAGL86)

The DEAE-Sepharose unbound fraction, as described above, was used for purification of 86-kDa α-glucosidase (ApAGL86). This fraction was subjected to phenyl-Sepharose chromatography. Most α-glucosidase activity was eluted with 1 M ammonium sulfate in 20 mM Tris–HCl (pH 7.0). After concentration, the phenyl-Sepharose unbound fraction was applied to a Sephacryl S-100 column. The fractions with α-glucosidase activity were concentrated and applied to a Mono Q HR5/5 column equilibrated with the same buffer and eluted with a linear gradient of NaCl (0–0.2 M) in the same buffer. The fraction possessing the activity was eluted as a single peak and contained an 86-kDa protein.

2.3.3. 59-kDa amylase (ApAmy59)

The G fraction eluted from phenyl-Sepharose [3] was concentrated and applied to a Sephacryl S-100 column (2.0 × 105 cm) equilibrated with 20 mM Tris–HCl (pH 7.0) containing 0.1 M NaCl. The G fraction was separated into two peaks (G-I and G-II). The second peak (G-II fraction) exhibited amylase activity and contained 59 kDa of protein as a major component.

2.3.4. 80-kDa amylase (ApAmy80)

The CM-Sepharose unbound fraction was applied to a DEAE-Sepharose (2.5 × 20 cm) column equilibrated with 20 mM Tris–HCl buffer (pH 7.0), and a linear gradient of NaCl (0–0.5 M), as described previously, was used to elute the bound proteins. Amylase activity was coeluted with 110-kDa β-glucosidase in DE-I fractions, as previously described [3]. The DE-I fraction was applied to a phenyl-Sepharose column and eluted using a linear gradient of ammonium sulfate (1–0 M). The fractions possessing amylase activity were concentrated by ultrafiltration and subjected to Sephacryl S-200 (3.0 × 95 cm) gel filtration and eluted with 20 mM Tris–HCl (pH 7.0) containing 0.1 M NaCl. The second peak exhibited α-amylase activity and contained an 80-kDa protein.

2.4. Analysis of degradation products by thin layer chromatography (TLC)

TLC for analysis of the degradation products of CMC, filter paper, lichenan, laminarin, disaccharide, and cello-oligosaccharides was performed on TLC Silica gel 60F plates (Merck KGaA, Darmstadt, Germany), and orcinol-sulfuric acid, as described previously, was used to detect the products [20].

2.5. Separation of degradation products by gel filtration

Reaction products were applied to a Bio-Gel P-2 column (1.2 × 81 cm, Bio-Rad Laboratories, CA, USA) equilibrated with water. Products were eluted with water at a flow rate of 10 ml/h, and 1.3-ml fractions were collected. The amount of reducing sugar

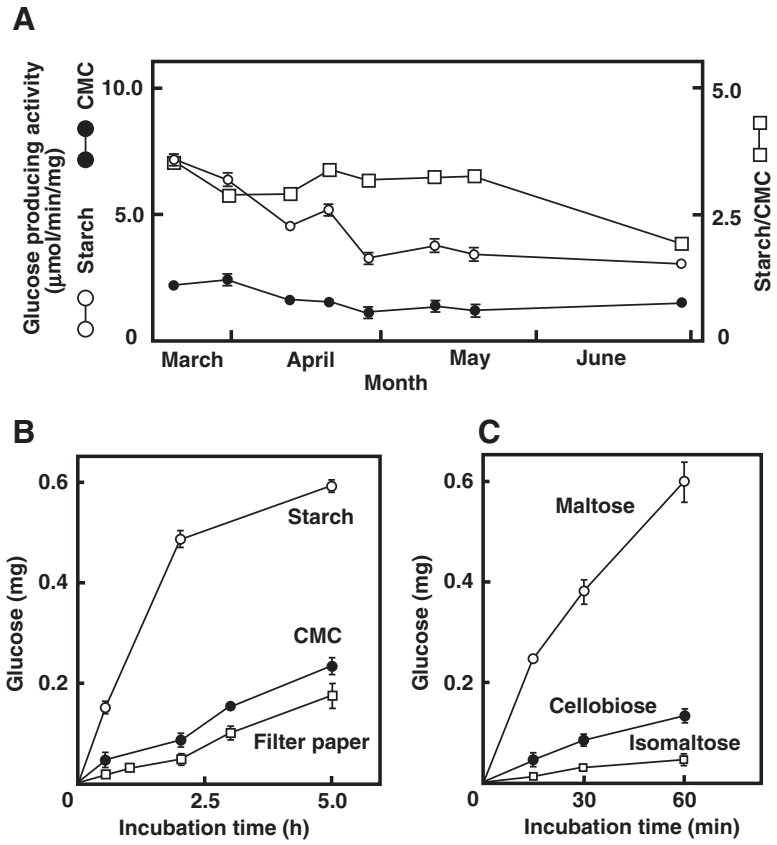

Fig. 1. Seasonal changes in the glucose-producing activities from starch and carboxymethylcellulose (CMC) in the digestive fluid of *Aplysia kurodai*. (A) Gastric digestive fluid was collected from ten *A. kurodai* on March 9 and 30; April 13, 20 and 28; May 11 and 18; and June 30 in 2013. Digestive fluids from three to four sea hare were combined and the enzyme activities in the three groups of digestive fluid were assayed. The digestive fluid (5 μl) was incubated in 0.2 ml of 1% starch or 1% CMC in 50 mM acetate buffer, pH 5.5, at 37 °C for 10 min. The reaction was terminated by heat treatment (95 °C, 5 °min), and glucose liberated from starch and CMC was determined by the Glucose CII Test Wako kit using glucose oxidase. Glucose-producing activity and ratio of activity towards starch and CMC were calculated from at least three separate experiments. Comparison of the glucose-producing activities from starch, CMC, filter paper (B), and maltose, cellobiose, and isomaltose (C) in the digestive fluid of *A. kurodai* collected on March 23. One percent starch, CMC, maltose, cellobiose, and isomaltose (0.2 ml in 50-mM acetate buffer at pH 5.5) were incubated with 2 μl of the digestive fluid (collected on Apr 13), and liberated glucose was determined. Filter paper (50 mg) was incubated with 2 μl of the same digestive fluid. Enzyme activity (mean ± S.D.) was calculated from at least three separate determinations.

and glucose in the fractions were determined by the Nelson and Somogyi method [17] and Glucose CII Test Wako.

2.6. Sequence analysis

Purified enzymes separated by SDS-PAGE were electroblotted onto PVDF membranes (Immobilon™, 0.45 mm, Millipore, Bedford, MA) according to the manufacturer's instructions. The protein band was stained with Ponceau 3R. For the determination of internal sequences, the protein band was digested by using lysyl endopeptidase [21], and the released peptides were purified by reversed-phase high-performance liquid chromatography, as described previously [22]. An automated protein sequencer (Shimadzu PPSQ-10, Kyoto, Japan) was used to analyze amino acid sequences.

2.7. Analysis of glycoprotein by lectin blot

Lectin blot of glycoprotein was performed by using horseradish peroxidase-labeled lectin (ConA, LCA, PHA-E4, PNA, RCA120, and WGA) according to the manufacturer's protocol. Purified amylases and α-glucosidases were blotted onto a PVDF membrane after SDS–PAGE, and the membrane was incubated with 20 μg/ml of lectin for 1 h at room temperature. A lectin-reactive band was then detected by using 3,3′-diaminobenzidine tetrahydrochloride.

3. Results

3.1. Starch and cellulose hydrolysis activities in the digestive fluid of A. kurodai

We first examined the glucose-producing activity from starch and cellulose in the digestive fluid of *A. kurodai*. In the Tokushima area, *A. kurodai* appear near seashores in March and grow by feeding on sea lettuce. The spawning season is in May and June. As shown in Fig. 1A, the glucose-producing activity in the digestive fluid is 3–5-fold higher from starch than from carboxymethylcellulose (CMC) in all months tested. The glucose-producing activities from starch and CMC were highest at the beginning of spring and decreased gradually. The glucose-producing activity was also lower from paper filter than from starch (Fig. 1B). Maltase activity was higher than cellobiose hydrolase and isomaltase activities (Fig. 1C). These findings suggested that *A. kurodai* uses starch in seaweed as an energy source.

3.2. Purification of 59-kDa (ApAmy59) and 80-kDa (ApAmy80) amylases

Two amylases, ApAmy59 and ApAmy80, were purified from the digestive fluid of *A. kurodai*, as described in Section 2. As shown in Fig. 2A, both amylases yielded single protein bands corresponding to molecular masses of 59 kDa and 80 kDa, respectively. Recently, 58-kDa (HdAmy58) and 82-kDa amylases (HdAmy82) were purified from the digestive fluid of Pacific abalone [9]. The two amylases from both species were purified by similar procedures, including hydrophobic, anionic ion-exchange, and hydroxyapatite column chromatography. The amino-terminal sequences of ApAmy59 and ApAmy80 were highly homologous with those of the 58 kDa and 82 kDa amylases of Pacific abalone, respectively (Fig. 2C). These sequence data strongly suggest that ApAmy59 and ApAmy80 are orthologs of abalone 58-kDa and 82-kDa α-amylase, respectively. The sequence, Phe-Glu-Trp located between the N-terminal α-helix and β-strand of salivary and pancreatic amylases was conserved in ApAmy59 (amino acid residues 17–19) [23]. The amino-terminal sequences of ApAmy59 and ApAmy80 were highly homologous with the corresponding regions of the protein sequences of the *A. californica* genome sequence that were predicted by automated computational analysis. The amino-terminal sequences of ApAmy59 and ApAmy80 were highly homologous with the corresponding sequences of α-amylase-like proteins, identified as NCBI reference sequence XP_005103373 (amino acid residues, 19–38; amino acid identity, 95%) and

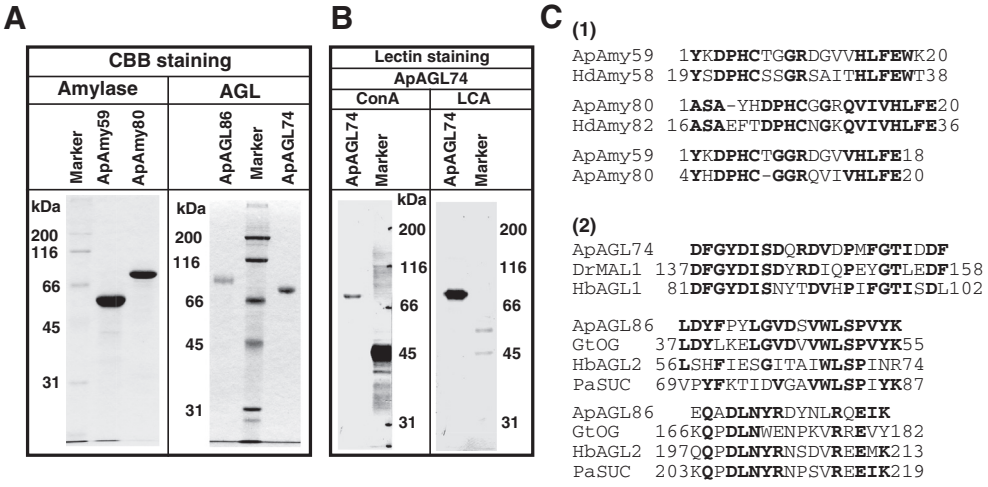

Fig. 2. SDS–PAGE and amino acid sequences of the purified amylases (ApAmy59 and ApAmy80) and α-glucosidases (ApAGL74 and ApAGL86). (A) Purified enzyme was boiled at 95 °C in 1% SDS solution containing 10% β-mercaptoethanol and then resolved by electrophoresis through 10% gel. Protein was detected using Coomassie Brilliant Blue. (B) Purified 74-kDa α-glucosidase (20 ng) was resolved by electrophoresis in 10% gel. Protein was detected by horseradish peroxidase-labeled lectin (ConA and LCA), as described in Section 2. Marker proteins comprised myosin heavy chain (200 kDa), β-galactosidase (116 kDa), BSA (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa). (C) Alignment of (1) N-terminal (ApAmy59 and ApAmy80) and (2) internal sequences (ApAGL74 and ApAGL86) of *Aplysia kurodai* enzymes with 58-kDa amylase (UniProtKB, L8AW48) and 82-kDa amylase (UniProtKB: L8AXN1) from the Japanese abalone, *Haliotis discus hannai*; maltase DrMAL1 from *Drosophila virilis* (UnitProtKB, O16098); α-glucosidase HbAGL1 (UnitProtKB, Q17058) and HbAGL2 (UniProtKB, Q25BT8) from honey bee, *Apis mellifera*; oligo-1,6-glucosidase GtOG from *Geobacillus thermoglucosidasius* (UnitProtKB, P29094); and sucrase PaSUC from pea aphid, *Acrythosiphon pisum* (UniProtKB, Q0H3F1). The amino-terminal sequence of amylases and internal sequences of fragments generated by lysyl endopeptidase digestion of purified ApAGL74 and ApAGL84 were determined, as described in Section 2. The amino acid residue numbers of *A. kurodai* amylases and other enzymes are indicated on both sides of the corresponding sequences.

XP_005089737 (amino acid residues, 18–37; amino acid identity, 90%), respectively. The specific activities of purified ApAmy59 and ApAmy80 were 4.15 and 73.8 U/mg, respectively. Approximately 6 mg of ApAmy59 and 0.4 mg of ApAmy80 were obtained from 300 ml of the digestive fluid of *A. kurodai*.

3.3. Purification of 74-kDa (ApAGL74) and 86-kDa (ApAGL86) α-glucosidases

Two α-glucosidases (ApAGL74 and ApAGL86) were identified in the digestive fluid of *A. kurodai*. ApAGL74 was eluted in the CM-Sepharose unbound fraction and further purified by employing a series of column chromatographic procedures using DEAE-Sepharose, Sephacryl S-100, hydroxyapatite (Fig. S1). Finally, ApAGL74 was purified to a homogeneous state by hydroxyapatite column chromatography. The activity was eluted in unbound and bound fractions of hydroxyapatite (Fig. S1D). α-Glucosidase in the unbound fraction yielded a single protein band on SDS–PAGE (Fig. S1E). The molecular mass of the enzyme was estimated to be 74 kDa by SDS–PAGE (Fig. 2A). The bound fraction contained another protein band (210 kDa) in addition to the 74-kDa protein; therefore, the 74-kDa α-glucosidase eluted in the unbound fraction was used as the purified enzyme. The specific activity of purified ApAGL74 against 4MU-α-glucoside was 20.0 U/mg. ApAGL86 was isolated from the DEAE-Sepharose unbound fraction and chromatographed on a phenyl-Sepharose column (Fig. S2A). The activity eluted in the phenyl-Sepharose unbound fraction was further purified on Sephacryl S-200 and Mono Q (Fig. S2B and C). On MonoQ chromatography, the enzyme activity was correlated with an 86-kDa protein band on SDS–PAGE (Fig. S2D). The fractions with activity were further purified by Mono Q rechromatography. The final preparation showed a single band on SDS–PAGE under a reducing condition (Fig. 2A). The molecular mass of the enzyme was estimated to be 86 kDa by SDS–PAGE, and the specific activity of the final preparation was 70.1 U/mg. Approximately 3.5 mg of ApAGL74 and 0.7 mg of ApAGL86 were obtained from 300 ml of the digestive fluid of *A. kurodai*.

In an effort to characterize ApAGL74 and ApAGL86 at the amino acid sequence level, the N-terminal sequences of the purified enzymes were examined. Approximately 100 pmol of ApAGL74 was applied to a protein sequencer, but no amino acid sequence was obtained, which suggested that the N-terminus of ApAGL74 was blocked. Consequently, the sequence of peptide #27 generated by lysyl endopeptidase digestion was determined. As shown in Fig. 2C, the internal sequence DFGYDISDQRDVDPMFGTIDDF of ApAGL74 has a high degree of homology to the corresponding regions of maltase (amino acid identity: 68%) from fruit fly [24] and α-glucosidases from honeybee (68%) [25]. Insect α-glucosidases are found exclusively in family GHF13 [26,27]. Although the N-terminus sequence (IDGQPVEYSPDPSEILTW) of ApAGL86 could be determined, enzymes containing the sequences homologous with the N-terminal sequence of ApAGL86 were not found in glycosidase-related enzymes. In contrast, the internal sequences #33 (LDYFPYLGVDSVWLSPVYK) and #41 (EQADLNYRDYNLRQEIK) of ApAGL86 were found to be homologous with the corresponding region of oligo-1,6-glucosidase from *Geobacillus thermoglucosidasius* [28], α-glucosidase from honeybee [29], and sucrase from pea aphid [30]. Particularly, the internal sequence (#33) of ApAGL86 showed the highest homology with the corresponding region of oligo-1,6-glucosidase from *G. thermoglucosidasius* (amino acid identity: 79%) [28]. The sequences, GVDSVWLSP (#33) and QADLN (#41) were highly conserved in CSR (conserved sequence region) VI and V of α-amylase family GHF13, respectively [27]. CSR VI and CSR V cover the strand β2 of the catalytic $(\beta/\alpha)_8$-barrel and domain B of GHF13, respectively. These results suggested that ApAGL74 and ApAGL86 belong to GHF13. In contrast, the internal sequences of ApAGL74 and ApAGL86 were highly homologous with the corresponding regions of the protein sequences of the *A. californica* genome sequence that were predicted by automated computational analysis. The internal sequence #27 of ApAGL74 was exactly the same as the corresponding region (87–108) of maltase 1-like protein (NCBI Reference sequence, XP_005107118). The internal sequence (#33) of ApAGL86 completely corresponded with the amino acid residue numbers 102–120 of α-glucosidase-like protein (NCBI Reference sequence, XP_005105339). The internal sequence (#41) was highly homologous (88% amino acid identity) with the corresponding region (187–203) of this α-glucosidase-like protein.

Recently, a 97-kDa α-glucosidase (HdAgl) was purified from the digestive fluid of Pacific abalone, and its cDNA was cloned [31]. Sequences homologous with the internal sequences of *A. kurodai* α-glucosidases were not found in the whole HdAgl sequence.

3.4. Analysis of oligosaccharides bound to the enzyme

To determine whether ApAmy59, ApAmy80, ApAGL74, and ApAGL86 are glycoproteins, the enzymes were analyzed by lectin blot. Six lectins comprising concanavalin A (ConA), peanut agglutinin (PNA), lentil agglutinin (LCA), wheat germ agglutinin (WGA),

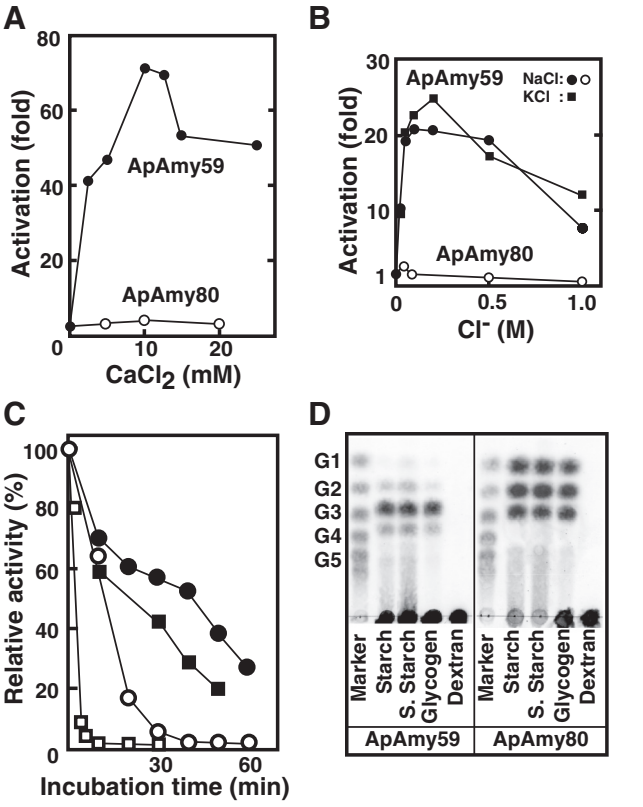

Fig. 3. Comparison of the enzymatic properties of ApAmy59 and ApAmy80. (A) Effect of $CaCl_2$ on the activities of ApAmy59 and ApAmy80. Amylase activities of the enzymes were assayed in the absence and presence of $CaCl_2$ as indicated. (B) Effect of NaCl and KCl on amylase activities of ApAmy59 and ApAmy80. Amylase activities of the enzymes were assayed in the absence and presence of NaCl and KCl as indicated. (C) Effect of $CaCl_2$ on heat stability of ApAmy59. ApAmy59 (35 μg/ml in 50 mM acetate, pH 6.0) was incubated in the absence (opened circles and squares) and presence of 10 mM $CaCl_2$ (closed circles and squares) at 45 °C (circles) and 50 °C (squares). After heat treatment, the enzyme solution was cooled in an ice bath, and amylase activity was determined in the presence of 10 mM $CaCl_2$. (D) Mode of action of ApAmy59 and ApAmy80 on starch, glycogen, and dextran. Starch, soluble starch, glycogen, and dextran (0.05 ml, 20 mg/ml in 50 mM acetate, pH 6.5, containing 10 mM $CaCl_2$) were incubated with 0.1 U of ApAmy59 and ApAmy80 at 37 °C for 15 h and subjected to TLC, as described in Section 2.

caster bean agglutinin (RCA-120), and phytohemagglutinin (PHA) were used. ConA, LCA, and WGA reactive oligosaccharide were detected in ApAGL74 (Fig. 2B). ApAmy59, ApAmy80, and ApAGL86 did not react with any lectins examined.

3.5. Characterization of ApAmy59 and ApAmy80

The specific activity of ApAmy59 (4.15 U/mg) was markedly lower than that of ApAmy80 (73.8 U/mg), abalone amylases, HdAmy58 (45 U/mg), and HdAmy82 (29 U/mg). Calcium and chloride ions have important roles in α-amylase activity and stability [32–35]. We examined the effects of the Ca^{2+} and Cl^- ion on the activity of ApAmy59. Among the metal ions examined, Ca^{2+} was found to be the strongest activator of ApAmy59. As shown in Fig. 3A, the specific activity of ApAmy59 increased about 70-fold (282 U/mg) by 10 mM $CaCl_2$. Although ApAmy59 was also activated by 10 mM $MgCl_2$ (13-fold), the effect was less than that by $CaCl_2$. $CoCl_2$ and $ZnCl_2$ possessed negative effects on the activity of ApAmy59. Similarly, Cl^- ion also activated ApAmy59 (Fig. 3B). ApAmy59 was activated maximally by either 0.2 M NaCl (25-fold) or 0.1 M KCl (21-fold). ApAmy59 activity in the presence of 10 mM $CaCl_2$ was not activated further by addition of NaCl. In contrast, ApAmy80 activity was not affected significantly by Ca^{2+} and Cl^-, as observed for ApAmy59. We next examined the Ca^{2+} binding activity of ApAmy59. ApAmy59 was incubated with 50 mM acetate (pH 6.0) containing 10 mM $CaCl_2$ at 0 °C for 15 h, and the activity was examined in the absence and presence of 10 mM $CaCl_2$. When the enzyme activity was assayed in the absence of Ca^{2+}, ApAmy59 activity was 1/20 relative to the activity in the presence of Ca^{2+}. These results indicated that the Ca^{2+} binding activity of ApAmy59 was very weak.

The optimal pH ranges for ApAmy59 and ApAmy80 were pH 6.0–6.5 and 5.5–6.0, respectively. The optimal temperatures for ApAmy59 and ApAmy80 were 40 °C and 55 °C, respectively. Both enzymes were stable at pH 5.0–7.0. The heat stability of ApAmy59 was increased by Ca^{2+} (Fig. 3C). Although ApAmy59 was completely inactivated by treatment at 50 °C for 10 min, ApAmy59 treated in the presence of Ca^{2+} retained 60% of the activity. The heat stability of ApAmy80 was not affected by the addition of Ca^{2+}. The *km* values of starch for ApAmy59 and ApAmy80 were 0.37 ± 0.03 and 1.42 ± 0.06 mg/ml in the presence of 10 mM $CaCl_2$. When the enzyme was incubated in the absence of $CaCl_2$, the *km* of starch for ApAmy59 increased 9.5-fold (3.50 ± 0.3 mg/ml). The *km* for ApAmy80 was not affected by $CaCl_2$. The V_{max} values for ApAmy59 in the absence and presence of $CaCl_2$ were 38.3 ± 2.4 U/mg and 370.4 ± 28.7 U/mg, respectively, and the values increased 9.7-fold by $CaCl_2$. The V_{max} value (101 ± 12.1 U/mg) for ApAmy80 increased 2-fold by $CaCl_2$. Thus, the presence of Ca^{2+} in the reaction mixture is required for expression of the maximum activity of *A. kurodai* ApAmy59, whereas Ca^{2+}-dependency of abalone HdAmy58 was not reported [9]. Fig. 3D shows the reaction products of starch, glycogen, and dextran by ApAmy59 or ApAmy80 digestion. ApAmy59 digested starch and glycogen and produced maltotriose as a major product, whereas maltotriose, maltose, and glucose were produced by ApAmy80, which indicated distinct cleavage specificity. Neither ApAmy59 nor ApAmy80 digested dextran.

To investigate the mode of hydrolysis of maltoheptaose and starch, the ApAmy59 and ApAmy80 degradation time courses of these substrates were analyzed (Fig. 4). As a control, the action of α-amylase from *Aspergillus oryzae* was also examined. All ApAmy59, ApAmy80, and *A. oryzae* amylase degraded maltoheptaose and starch and produced maltotriose, maltose, and glucose. Trace amounts of oligosaccharides longer than maltotriose were also detected in digestion of starch. The ratio of final major products differed. The relative amounts of reaction products of maltoheptaose

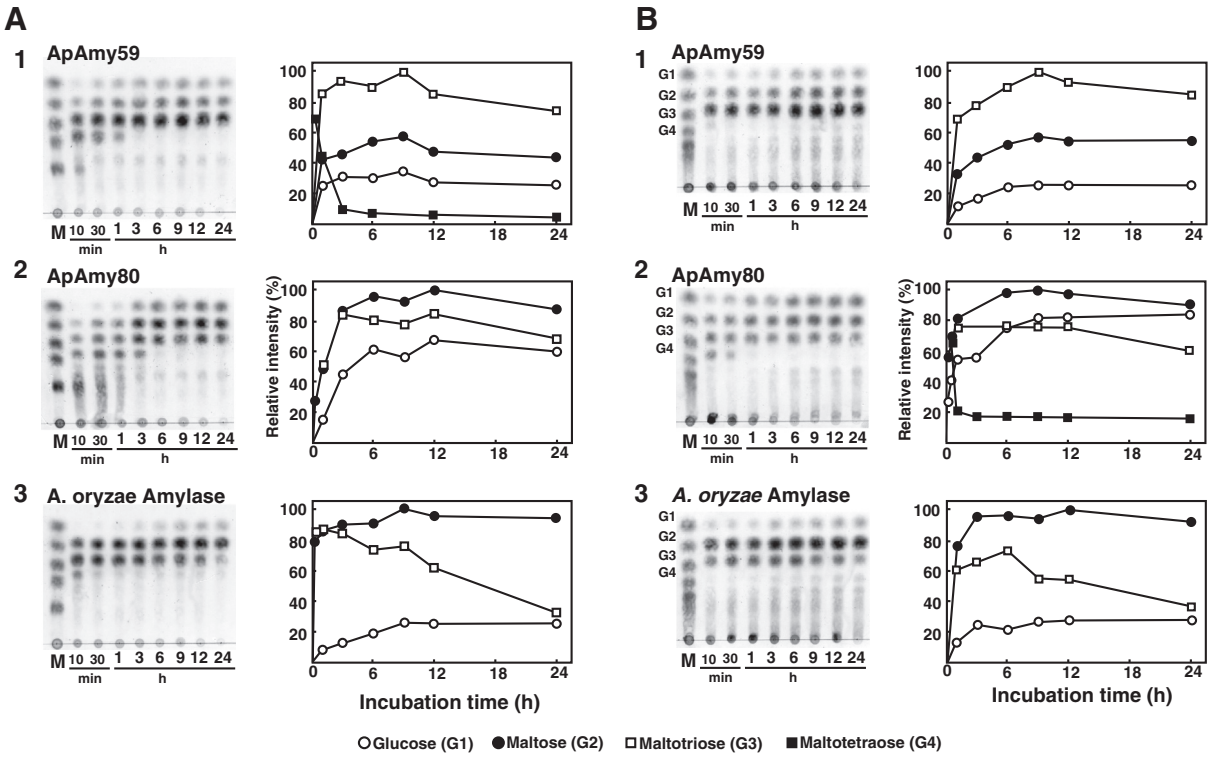

Fig. 4. Degradation of maltoheptaose and starch by ApAmy59 and ApAmy80. Maltoheptaose (A) and starch (B) (0.2 ml, 10 mg/ml in 10 mM acetate, pH 6.0, containing 10 mM $CaCl_2$) were incubated with 0.18 U of ApAmy59 and ApAmy80 at 37 °C for the time indicated. Fungal amylase from *Aspergillus oryzae* was incubated in the absence of $CaCl_2$. The reaction was terminated by heat treatment (95 °C for 2 min) and analyzed by TLC. The intensity of reaction products was analyzed by densitometer.

and starch incubated with ApAmy59 were maltotriose > maltose > glucose. ApAmy80 hydrolyzed maltoheptaose or starch and produced maltose > glucose ⩾ maltotriose. Although the major product of *A. oryzae* amylase was maltose as observed for ApAmy80, the glucose-producing activity was markedly lower for *A. oryzae* amylase than for ApAmy80. Thus, ApAmy80 possessed the highest glucose-producing activity from maltoheptaose and starch among the three enzymes tested. Both ApAmy59 and ApAmy80 did not hydrolyze maltose, cellobiose, CMC, laminarin, lichenan, and 4MU-α-glucoside.

3.6. Characterization of ApAGL74 and ApAGL86

ApAGL74 and ApAGL86 exhibited optimal hydrolytic activity towards 4MU-α-glucoside at pH 5.5–6.0. The optimal temperatures for ApAGL74 and ApAGL86 were 35 °C and 40 °C, respectively. Both enzymes were unstable at temperatures >45 °C and stable at pH 5.0–7.5. Hydrolysis of 4MU-α-glucoside by ApAGL74 and ApAGL86 was not inhibited by 0.2 M glucose. The substrate specificities of ApAGL74 and ApAGL86 were first compared briefly using TLC (Fig. 5A). ApAGL74 completely hydrolyzed maltose and maltoheptaose to produce glucose. Sucrose was also hydrolyzed by ApAGL74, whereas starch was not a good substrate for ApAGL74. In contrast, the hydrolysis activity of ApAGL86 against these natural substrates was not detected by TLC. Both enzymes exhibited no activity toward cellobiose, lactose, laminarin, lichenan, 4MU-β-glucoside, 4MU-α-galactoside, 4MU-α-mannoside, and 4MU-β-xyloside.

To investigate the mode of hydrolysis of maltoheptaose by ApAGL74, the time-course of degradation was analyzed (Fig. 5B). Maltoheptaose was completely hydrolyzed to glucose within 1 h. Trace amounts of maltose and maltotriose were detected. The *km* and *kcat* values of ApAGL74 for maltoheptaose were estimated to be 31.7 ± 2.1 mM and 40.2 ± 1.2 s^{-1}, respectively.

To compare the substrate specificities of ApAGL74 and ApAGL86 precisely, the *km* and *kcat* values toward 4MU-α-glucoside, maltose, isomaltose, sucrose, dextran, glycogen, and starch for ApAGL74 and ApAGL86 were determined (Table 1). Although α-glucosidase I from Japanese honeybee showed unusual kinetic features toward maltose, p-nitrophenyl-α-glucoside, and sucrose [36], ApAGL74 and ApAGL86 exhibited normal Michaelis–Menten-type kinetics for all substrates tested. Judging from the *kcat/km*, it is likely that ApAGL74 prefers short α-1,4-linked oligosaccharides. In contrast, ApAGL86 is likely to prefer long α-1,6 and α-1,4-linked polysaccharides, such as glycogen.

The synergistic effects of α-amylases and ApAGL74 on glucose production from starch were examined (Fig. 5C). Starch (2 mg) was incubated with ApAmy59, ApAmy80, ApAGL74, ApAmy59 + ApAGL74, ApAmy80 + ApAGL74, and ApAmy59 + ApAmy80 + ApAGL74 in 50 mM acetate (pH 6.5) containing 10 mM $CaCl_2$. Although glucose production from starch by reaction with amylase or β-glucosidase only was very low (ApAmy59, 0.12 mg glucose; ApAmy80, 0.2 mg; ApAGL74, 0.15 mg; after 8 h), glucose production was markedly increased by incubation with α-amylase and ApAGL74. After 8 h, 80% of starch was converted to glucose. When two types of α-amylases and ApAGL74 were incubated together, the glucose production rate was slightly increased. Next, the effect of ApAGL74 on glucose production from sea lettuce by α-amylase treatment was investigated (Fig. 5D). Sea lettuce is the primary food of sea hares. Approximately 0.6 mg of glucose was liberated from sea lettuce (20 mg dry weight) when 0.1 U of ApAmy59 or ApAmy80 was incubated in the presence of 0.02 U of ApAGL74. Further glucose production from sea lettuce by α-amylase from *A. oryzae* and ApAGL74 was also examined. Although the same amount of activity of fungal α-glucosidase was used, glucose production (0.2 mg) was markedly decreased compared with that from treatment with *A. kurodai* enzymes.

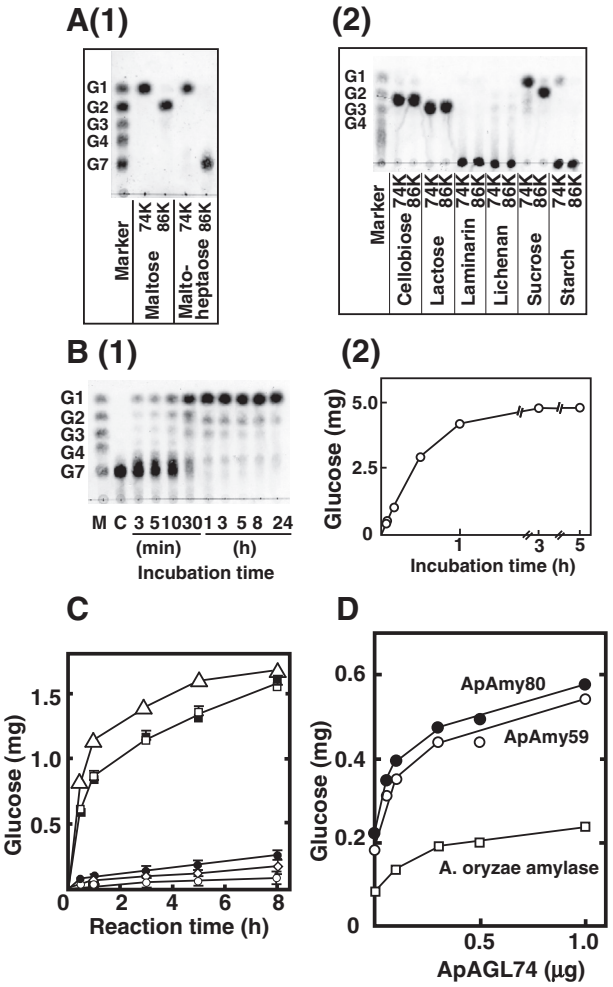

Fig. 5. Comparison of the enzymatic properties of ApAGL74 and ApAGL86. (A-1) Maltose and maltoheptaose (0.2 ml, 1 mg/ml in 10 mM acetate, pH 6.0) were incubated with 0.03 U of ApAGL74 or ApAGL86 at 37 °C for 24 h. (A-2) Cellobiose, lactose, laminarin, lichenan, sucrose, and starch (0.2 ml, 10 mg/ml in 10 mM acetate, pH 6.0) were incubated with 0.03 U of ApAGL74 or ApAGL86 at 37 °C for 24 h. Reaction products were analyzed by TLC. (B-1) Time-course hydrolysis of maltoheptaose by ApAGL74. ApAGL74 (0.1 U) was incubated with celloheptaose (1.0 ml, 5 mg/ml in 10 mM acetate, pH 6.0) at 37 °C for the time indicated. The reaction products were analyzed by TLC (B-1) and glucose content was determined by the Glucose CII Test Wako (B-2). (C) Hydrolysis of starch by the synergistic action of amylase and α-glucosidase. Incubation of 0.08 U of ApAmy59 and ApAmy80 with starch (1.0 ml, 2 mg/ml in 50 mM acetate, pH 5.5 containing 10 mM $CaCl_2$) in the absence or presence of ApAGL74 (0.08 U) at 37 °C for the time indicated. Glucose (mean ± S.D.) was determined by at least three separate experiments. ApAmy59 (opened circles), ApAmy80 (closed circles), ApAGL74 (open diamonds), ApAmy59 + ApAGL74 (opened squares), ApAmy80 + ApAGL74 (closed squares), and ApAmy59 + ApAmy80 + ApAGL74 (opened triangles). (D) Hydrolysis of sea lettuce by the synergistic action of amylase and α-glucosidase. Sea lettuce (1.0 ml, 20 mg/ml in 50 mM acetate containing 10 mM $CaCl_2$) was incubated with 0.1 U of ApAmy59 and ApAmy80 in the absence and presence of ApAGL74 at 37 °C for 24 h. Aspergillus amylase was incubated in the absence of $CaCl_2$. Glucose (mean) was determined by two separate experiments.

3.7. Glucose production from sea lettuce by ApAmy59, ApAmy80, and ApAGL74

To understand the physiological role of the digestive enzymes, we compared the glucose productivities between the cellulose digestion system and the starch digestive system from various seaweeds and microalgae (Fig. 6A). A pair comprising 45-kDa cellulase and 210-kDa β-glucosidase purified from the digestive fluid of *A. kurodai* [3] was used as the cellulose digestive system. In the digestive fluid of *A. kurodai*, 45-kDa cellulase and 210-kDa

Table 1
Substrate specificities of ApAGL74 and ApAGL86.

Substrates[a]	AGL	*Km*	*kcat*[b]	*kcat*/*Km*
4MU-α-glucoside		(μM)	(s^{-1})	
	ApAGL74	47.9 ± 10.6	27.1 ± 3.32	0.566
	ApAGL86	412 ± 6.0	3.02 ± 0.03	0.0073
Maltose		(mM)	(s^{-1})	
	ApAGL74	4.98 ± 0.06	89.0 ± 20.8	17.9
	ApAGL86	1.86 ± 0.11	3.83 ± 0.15	2.06
Isomaltose		(mM)	(s^{-1})	
	ApAGL74	14.2 ± 0.90	0.535 ± 0.02	0.0377
	ApAGL86	16.5 ± 0.05	24.5 ± 0.015	1.48
Sucrose		(mM)	(s^{-1})	
	ApAGL74	31.7 ± 2.10	40.2 ± 1.15	1.27
	ApAGL86	33.6 ± 1.55	11.7 ± 0.42	0.348
Dextran		(%)	(s^{-1})	
	ApAGL74	6.30 ± 1.31	0.348 ± 0.024	0.0552
	ApAGL86	5.38 ± 0.37	16.5 ± 0.98	3.07
Glycogen		(%)	(s^{-1})	
	ApAGL74	1.42 ± 0.20	0.592 ± 0.153	0.417
	ApAGL86	0.052 ± 0.005	2.93 ± 0.30	56.3
Starch		(%)	(s^{-1})	
	ApAGL74	0.819 ± 0.08	2.16 ± 0.14	2.64
	ApAGL86	0.098 ± 0.01	2.30 ± 0.14	23.5

[a] The kinetic data and SDs were calculated from at least three times of experiments.
[b] Based on a molecular mass of 74 kDa and 86 kDa.

β-glucosidase are the most abundant cellulase and β-glucosidase, respectively. A pair comprising ApAmy59 + ApAGL74 and ApAmy80 + ApAGL74 was used as the starch digestive system. Enzyme digestions of *U. pertusa* (sea lettuce), *U. prolifera*, *E. bicyclis*, *Saccharina* sp., and *C. vulgaris* were performed at 37 °C and pH 5.5, which is the pH of the digestive fluid of *A. kurodai* in the presence of 10 mM $CaCl_2$. When the algae were treated by the cellulose digestive system, the amount of glucose liberated was highest for *U. pertusa* and *U. prolifera* digestion. In contrast, the amount of glucose liberated from *U. pertusa* by the starch digestive system was markedly higher than that by the cellulose digestive system. *C. vulgaris* was also a good substrate for the starch digestive system, as was sea lettuce. Glucose was not produced from *E. bicyclis* by either the cellulose or starch digestive systems. Thus, a greater amount of glucose was produced from sea lettuce by amylase and α-glucosidase treatment than by digestion with cellulolytic enzymes.

In an effort to understand the synergistic effects of ApAmy59, ApAmy80, ApAGL74, and ApAGL80 on glucose production from sea lettuce, sea lettuce was incubated with various combinations of *A. kurodai* amylase and α-glucosidase, and then the reaction products were analyzed by gel filtration and TLC. Degradation products were detected in tube numbers 50–64 on gel filtration by measuring the amount of reducing sugar formed (Fig. 6B). ApAmy59 and ApAmy80 displayed distinct cleavage specificities toward starch in sea lettuce, as in the case of starch digestion. When sea lettuce was digested with ApAmy59, three spots corresponding to maltotriose, maltose, and glucose were detected. Maltose was the major spot. ApAmy80 hydrolyzed starch in sea lettuce to maltose and glucose. The addition of ApAGL74 resulted in an increase in glucose productivity. Thus, *A. kurodai* amylase can produce glucose from sea lettuce without α-glucosidase, and glucose yield was markedly increased by the combination of α-amylase and α-glucosidase. To identify the minimum combination of α-amylase and α-glucosidase for maximizing glucose productivity, the glucose-producing activities of the various enzyme mixtures toward sea lettuce were compared (Fig. 6C). Sea lettuce (20 mg/dry weight) was digested by various mixtures of purified enzymes (2 μg of enzyme) at 37 °C for 20 h. The reaction using three enzymes (ApAmy59, ApAmy80, and ApAGL74) gave approximately 84% of the glucose productivity obtained from the same reaction using all enzymes (1.05 mg of glucose production).

4. Discussion

In the present study, two α-amylases, ApAmy59 and ApAmy80, and two α-glucosidases, ApAGL74 and ApAGL86, were isolated from the digestive fluid of *A. kurodai*. The amino-terminal sequences of ApAmy59 and ApAmy80 were highly homologous with the amino-terminal sequence of the mature form of abalone 58-kDa (HdAmy58) and 82-kDa amylases (HdAmy82), respectively [9]. Sequence data strongly suggested that ApAmy59 and ApAmy80 are orthologs of HdAmy58 and HdAmy82, respectively. The two α-glucosidases possess sequences homologous with those of glycosidases belonging to GHF13 [5–7,26,27]. The internal sequence of ApAGL74 was homologous with that of the fruit fly maltase [24] and honeybee α-glucosidase [25]. The internal sequences of ApAGL86 were homologous with the corresponding region of *Geobacillus* oligo-1, 6-glucosidase [28], honeybee α-glucosidase 2 [29], and pea aphid sucrase [30]. In contrast, the amino terminal sequences of ApAmy59 and ApAmy80 and internal sequences of ApAGL74 and ApAGL86 were exactly the same or highly homologous with the corresponding sequence regions of the maltase 1-like (NCBI: XP_005107118), α-amylase-like (NCBI: XP_005105339), α-glucosidase-like (NCBI: XP_005103373), and α-glucosidase-like (NCBI: XP_005089737) gene products predicted by automated computational analysis of the *A. californica* genome sequence, respectively. These results strongly suggest that ApAmy59, ApAmy80, ApAGL74, and ApAGL86 are produced by *A. kurodai* itself and not by gut-resident bacteria and protists.

Enzymatic properties, including optimal pH, optimal temperature, and cleavage specificity of ApAmy59, were similar to those of HdAmy58; however, a remarkable difference in specific activity between ApAmy59 and HdAmy58 was found. The specific activity of ApAmy59 (4.15 U/mg) was remarkably lower than that of HdAmy58 (45 U/mg). As a consequence of screening of the ApAmy59 activator, Ca^{2+} and Cl^{-} were found to activate ApAmy59 activity markedly. The activity of ApAmy59 was activated to 70-fold and 25-fold by the addition of 10 mM $CaCl_2$ and 0.2 M NaCl, respectively. All known α-amylases belonging to a group of metalloenzymes contain a conserved calcium ion [32]. Removal of calcium from the enzyme results in decreased enzyme activity and stability [33,34]. Further, several α-amylases, including mammalian pancreatic and insect α-amylases, have been found to require chloride to show full catalytic activity [37,38]. In contrast, most microbial amylases are not affected by the presence of chloride. It is likely that the Ca^{2+} binding activity of ApAmy59 is very weak compared with those of other eukaryotic amylases. Considering the concentrations of Ca^{2+} (10 mM) and Cl^{-} (0.5 M) in sea water and the calcium content (4.9 mg/g dry weight) in sea lettuce, ApAmy59 is likely to be fully activated in the stomach of *A. kurodai*. ApAmy80 possesses higher glucose-producing activity from maltoheptaose, starch, and glycogen than does ApAmy59.

Compared with bacterial and fungal α-glucosidase reports, there are very few reports concerning enzymatic characterization of α-glucosidase purified from invertebrates. To date, invertebrate α-glucosidase has been purified from honeybee (*Apis cerana*) [39], (*A. mellifera*) [25,29], fruit fly (*Drosophila melanogaster*) [40], abalone (*Haliotis discus hannai*) [31], mottled sea hare (*A. fasciata*) [41], and shrimp (*Penaeus vannamei* and *P. japonicus*) [12,42]. These enzymes belong to GHF 13 but differ in substrate specificity and molecular mass. The best substrates for honeybee and shrimp

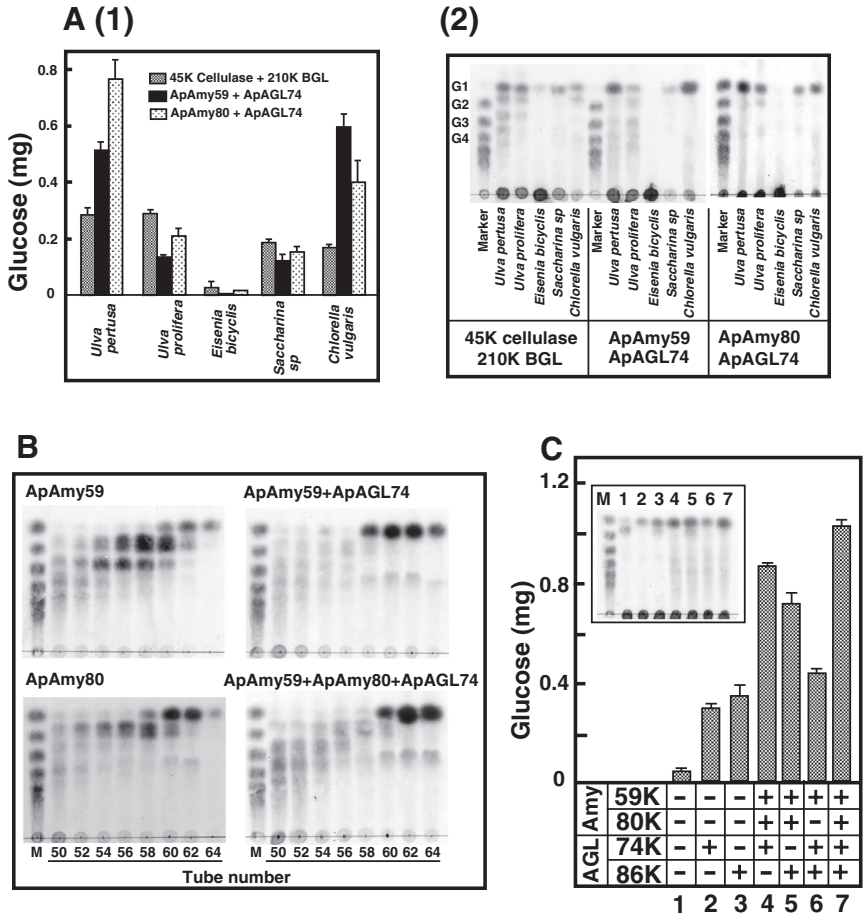

Fig. 6. Saccharification of algae with cellulase, β-glucosidase, amylase, and α-glucosidase purified from *A. kurodai*. (A) Macroalgae, sea lettuce (*Ulva pertusa*), green lavor (*U. prolifera*), *Eisenia bicyclis*, *Saccharina* sp. and the microalgae, *Chlorella vulgaris* (10 mg in 0.5 ml of 50 mM acetate, pH 5.5, containing 10 mM $CaCl_2$), were incubated with purified enzyme (5 μg) at 37 °C for 20 h. Glucose content was then determined (A-1). TLC analysis of reaction products of algae treated with purified enzymes (A-2). (B) Gel filtration analysis of reaction products of algae treated with amylase (ApAmy59 or ApAmy80) in the absence and presence of ApAGL74. Sea lettuce (50 mg in 1.5 ml of 50 mM acetate, pH 5.5, containing 5 mM $CaCl_2$) was incubated with amylase (20 μg) in the absence and presence of ApAGL74 (20 μg) as indicated at 37 °C for 24 h. After centrifugation, the supernatants were fractionated by gel filtration through a Bio-Gel P-2 column, as described in Section 2. Eluants (0.5 ml, tube number 50–64) were lyophilized and dissolved in 20 μl of H_2O. A 2-μl aliquot was used for TLC. (C) Sea lettuce (20 mg in 1.0 ml of 50 mM acetate, pH 5.5, containing 10 mM $CaCl_2$) was incubated with various mixtures of purified enzymes (2 μg) at 37 °C for 20 h. Glucose content (mean ± S.D.) in the supernatant of the reaction mixtures was determined by three separate experiments. TLC analysis of reaction products (inset).

α-glucosidase were shown to be sucrose and maltose, respectively. Sucrose was not hydrolyzed by shrimp α-glucosidase. The molecular masses of shrimp and honeybee α-glucosidase were estimated to be approximately 105 kDa [12] and 68 kDa [29], respectively. The 98-kDa abalone α-glucosidase (98-kDa HdAgl) preferably hydrolyzed smaller substrates such as maltose and maltotriose [31]. Starch was also hydrolyzed to glucose by the abalone enzyme; however, sucrose and isomaltose were not cleaved by abalone α-glucosidase. Its ortholog was not found in *A. kurodai*. Further analysis is necessary.

Although the molecular mass of α-glucosidases from *A. fasciata* (69 kDa) [41] and *A. kurodai* (ApAGL74, 74 kDa) were similar, their cleavage specificities clearly differed. The former enzyme hydrolyzed maltose and maltotriose but not starch and sucrose. ApAGL74 prefers smaller substrates, such as maltose; however, it can hydrolyze sucrose, unlike α-glucosidase from *A. fasciata*. In contrast, ApAGL86 is the first purified α-glucosidase from an invertebrate that prefers hydrolysis of the α-1, 6 glycoside bond of glucose. The *kcat/km* values for ApAGL86 towards isomaltose, dextran, and glycogen were 39, 56, and 135-fold higher than those for ApAGL74, respectively.

In an effort to understand the seaweed starch digestive system of *A. kurodai* and identify the best combination of amylases and α-glucosidases to maximize glucose productivity, the glucose-producing activities of the various enzyme mixtures on sea lettuce were investigated. Sea lettuce is the best substrate for *A. kurodai* α-amylase and α-glucosidase. When 20 mg of sea lettuce was digested by four enzymes, ApAmy59, ApAmy80, ApAGL74, and ApAGL86, at 37 °C for 24 h, approximately 1 mg of glucose was produced. However, almost the same amount of glucose (0.8 mg) was produced even by reaction with ApAmy59, ApAmy80, and ApAGL74 without ApAGL86. These results suggested that sea lettuce contains an amylose-type starch. ApAGL86 might have a role in digestion of amylopectin-type starch. In contrast to the amylolytic system, the sea hare cellulolytic system comprised four endo-β-1,4-glycosidases (95-kDa, 65-kDa, 45-kDa and 21-kDa cellulases) and two β-glycosidases (210-kDa and 110-kDa β-glucosidases) [3]. The core components of the sea hare cellulose digestive system are a 45-kDa cellulase and a 210-kDa β-glucosidase. The starch and cellulose concentrations in sea lettuce (*U. pertusa Kjellman*) were estimated to be 20% and 8%, respectively [4]. It is noteworthy that approximately a 3- to 5-fold greater amount of glucose was produced from starch by incubation with the digestive fluid of sea hare relative to that from CMC. The specific activity of ApAmy59 (282 U/mg in the presence of Ca^{2+}) toward starch is 16.5-fold higher than the specific activity of 45-kDa cellulase

(17.1 U/mg), which is the most abundant cellulase in the digestive fluid, toward CMC. The *km* value of starch for ApAmy59 (0.37 ± 0.03 mg/ml) is markedly lower than the *km* of CMC for 45-kDa cellulase (81.3 ± 14.0 mg/ml). Thus the higher concentrations of starch relative to those of cellulose in sea lettuce and the higher catalytic efficiency of the amylolytic system relative to that of the cellulolytic system may lead to better digestion of starch. It is highly likely that starch in sea lettuce is a predominant glucose source for sea hare.

In mammals, two membrane-bound enzyme complexes, maltase–glucoamylase and sucrase–isomaltase belonging to GHF31, hydrolyze starch-derived products to glucose monomer in the small intestine [13,14]. Previously, we showed the similarity of *A. kurodai* 210-kDa β-glucosidase with human intestinal lactase-phlorizin hydrolase in terms of molecular mass, amino acid sequences, and catalytic properties [3]. In contrast, the corresponding region homologous with the internal amino acid sequences of the two *A. kurodai* α-glucosidases are not found in sequences of maltase–glucoamylase and sucrase–isomaltase. The sequences of *A. kurodai* α-glucosidases are highly homologous with invertebrate or bacterial α-glucosidases belonging to GHF13.

In conclusion, our work provides the first comprehensive analysis of the digestive amylolytic system of *A. kurodai*. Our results clearly showed that *A. kurodai* can digest starch and produce glucose efficiently by the combination of two α-amylases and two α-glucosidases in the stomach, suggesting that 74-kDa and 86-kDa α-glucosidases can substitute for the maltase–glucoamylase and sucrase–isomaltase complexes. Further, the starch digestive system of *A. kurodai* may contribute important insights into the development of biofuel processing of seaweed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.06.002.

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