Fish Sci (2014) 80:589–601
DOI 10.1007/s12562-014-0708-z

ORIGINAL ARTICLE

Chemistry and Biochemistry

Analysis of the α-*amylase* gene sequence and the enzyme activity of Indian rock oyster *Saccostrea forskali*

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Received: 24 September 2013 / Accepted: 19 January 2014 / Published online: 14 February 2014

Abstract Indian rock oyster *Saccostrea forskali* is an important commercial species in Thailand. In this study, its full-length α-*amylase* (*SfAmy*) cDNA nucleotide sequence was investigated. The *SfAmy* cDNA was 1,689 bp long and contained a 1,563-bp open reading frame encoding 520 amino acid residues, including a 17-amino acid signal peptide. The molecular mass and the estimated isoelectric point (p*I*) of the deduced mature *S. forskali* α-amylase (SfAMY) were 55.948 kDa and 6.45, respectively. The deduced protein sequence showed 45–88 % identity to other mollusk AMYs. The molecular weight was confirmed by the weight of the purified native enzyme. The specific activities of crude and purified native enzymes toward 1 % starch were 29.53 and 187.42 U/mg. In addition, the obtained recombinant SfAMY also showed activity in digesting 1 % starch. The specific activities of the crude and purified recombinant proteins were 11.8 and 46 U/mg. Both enzymes showed optimal activity temperature at 40 °C but their optimum pH values were different, 6.0 for the native and 5.0 for the recombinant. The expression of *SfAmy* examined by RT-PCR showed the highest levels in the digestive gland but none was observed in the adductor muscle.

Keywords α-Amylase · Mollusk · Indian rock oyster · *Saccostrea forskali*

Introduction

α-Amylases (α-1,4-glucan-4-glucanohydrolases) are enzymes that catalyze the hydrolysis of the α-(1,4) glycosidic linkages in starch and related compounds. With their main function in carbohydrate metabolism, these enzymes are important for the utilization of energy sources in animals, higher plants and microorganisms. Moreover, these enzymes have become important industrial enzymes [1, 2]. Thus, α-amylases from bacteria to mammals have been characterized both biochemically and molecularly. The α-*amylase* genes encoding α-amylase enzymes have been cloned and characterized in a number of organisms, including animals such as insects [3, 4], chickens [5], shrimps [6], fishes [7], pigs [8], and humans [9]. For mollusks, the sequences of α-*amylase* genes were firstly determined from *Pecten maximus* [10], and later from other species such as *Crassostrea gigas* [11], *Corbicula fluminea* [12], *Haliotis discus discus* [13], *Pteria penguin* (Accession Number JF748721), and *Haliotis discus hannai* [14].

All α-amylases belonging to the glycoside hydrolase family 13 are characterized by the presence of three domains: A, B and C. Domain A contains a (β/α) eight-barrel domain, with domain B inserted between the third β strand and the third α helix. Domain C is a C-terminal eight-strand β sheet [15–17]. Although all α-amylases have

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the same functions and their tertiary structures are strongly conserved, their amino acid and nucleotide sequences vary quite a lot among different groups. For example, the identity of α-amylase amino acid sequences among the inter-kingdom groups of microorganisms, plants and mammals, could be less than 10 % [18, 19]. However, all α-amylases contain invariable amino acid residues at four positions, Arg (R) in the β3 sheet and three catalytic residues, Asp (D) in the β4 sheet, Glu (E) in the β5 sheet and Asp (D) in the β7 sheet [20]. In animals, the amino acid sequences of α-amylases show several conserved regions that are typical of animal α-amylases. All animal α-amylases are chloride-dependent so the amino acid residues for the chloride binding sites are conserved [17, 21]. Moreover, eight cysteine residues forming four disulfide bonds are conserved in all animal α-amylases [21].

Indian rock oyster *Saccostrea forskali* is a local oyster species that is commonly found along the coast of Thailand. The oyster has been cultured in the east of the Gulf of Thailand for over 50 years since it is a popular sea food and thus represents a high income for the farmers. Currently, in Thailand, it is an economically important oyster [22]. For cultivation, spats are collected from nature and taken to a farm set in the sea. The spats grow naturally when fed with both animal and plant sea planktons. Even though, at present, there is no supplementary or commercial food available for oysters, their digestive systems should be studied. So far, the major digestive enzyme found in most bivalves is α-amylase [10, 23]. In this paper, the full-length *α-amylase* cDNA (*SfAmy*) sequence of *S. forskali* and the deduced protein (SfAMY) sequence were analyzed. The molecular weight and activity of the purified native and recombinant SfAMY were also studied.

Materials and methods

Oyster

Mature Indian rock oysters *S. forskali* of 80–120 g were purchased from a farm in Cholbury province, in the eastern part of Thailand.

RNA isolation and cDNA sequence

Total RNA was extracted from the digestive gland of the oyster using TRIzol reagent (Gibco BRL, USA). First strand cDNA was synthesized using Reverse Transcription System Kit (Promega), according to the manufacturer's instructions. The primers (Table 1) were designed based on the *α-amylase* (*amy*) gene sequences of other mollusks available on the NCBI database. The first strand cDNA was used as a template to amplify the *α-amylase* cDNA with the primers, AmyF and AmyR (Table 1), under the following conditions: preheated at 94 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 56 °C for 45 s and extending at 72 °C for 1 min, and final extension at 72 °C for 10 min. The obtained PCR product was cloned into the pGEM^{T} Easy plasmid vector and sent for sequencing at Macrogen, Korea. The obtained cDNA sequence was BLASTed to the NCBI database using Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to check that it was an *amy* gene sequence.

Table 1 Primers used in this study

Primer names	Sequences (5′ → 3′)	Product length (bp)
AmyF	CCGGYGTCCCTTTYTCTTCCTGG	650
AmyR	GCGATGGCGTAGTYACCRTTGTCCC	
Amy5′ RACE	GGCCCTGCCAGTTATTGCGATCCTCCCC	1,100
Amy3′ RACE	CGGAGTTCATGTGGCGCATCCTTACGG	700
β-actinF	GACGCCCCAGACATCAGGGT	670
β-actinR	GTGATGACCTGACCGTCGGG	
RT-AmyF	GGGCACACGTGTCAGTACA GCGTT	620
RT-AmyR	AAGCCAGACCCTTGCTTGCTCCTC	
AMY-F	TCTAGAGGTACGTGGAGTAACCCG	1,512
AMY-R	CATATGCTACGTTGTGACTTTCTTTG	
UMP[a]	CTAATACGACTCACTATAGGGC	

[a] *UMP* universal primer of SMARTM RACE cDNA Amplification Kit

5′, 3′ RACE for generating the full-length cDNA

To obtain the full-length *SfAmy* cDNA sequence, both 5′ and 3′ rapid amplifications of cDNA ends (RACE) were conducted using the Smart cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions. The gene-specific primers, Amy 5′ RACE and Amy 3′ RACE (Table 1) were designed based on the *SfAmy* cDNA sequence obtained. The 5′-RACE PCR and 3′ RACE PCR fragments were cloned and sequenced. The three PCR sequences obtained were assembled to get the full-length *SfAmy* cDNA sequence using CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php).

Sequence alignment and phylogenetic analyses

The full-length *SfAmy* cDNA sequence was translated into the deduced amino acid sequence using Expasy-Translation tool (web.expasy.org/translate/). The deduced amino acid sequence obtained was then aligned with some known α-amylase (AMY) protein sequences which are available on the NCBI database (the NCBI database: http://blast.

ncbi.nlm.nih.gov/Blast.cgi "Accessed 15 Oct 2013") using the ClustalW version 2.0.10. Phylogenetic trees based on AMY protein sequences were constructed using maximal likelihood methods, MEGA version 5.05 [24]. The reliability of clusters within the tree was evaluated based on 1,000 bootstrap replications.

Expression analysis using RT-PCR

Total RNAs were extracted from several tissues of *S. forskali* including digestive gland, labia palps, gills, mantle, and adductor muscle, using TRIzol reagent (Gibco BRL, USA). A portion of 3 μg of the total RNA of each tissue was used for reverse transcriptase polymerase chain reactions (RT-PCR) using the Reverse Transcription System Kit (Promega, USA) to get the cDNAs of these tissues. RT-PCRs were performed using the cDNAs as templates and with RT-AmyF and RT-AmyR (Table 1). As the control, β-*actin* gene was amplified using the primers β-actinF and β-actinR (Table 1) which were designed based on *C. gigas β-actin* gene sequence (Accession Number EU234531). The amplification was carried out in a total volume of 30 μl, which included 1 μl of cDNA, 1.5 μl of each primer (10 mM), 2 μl of dNTP (2.5 mM), 0.1 μl of *Taq* DNA polymerase, and 21.9 μl of distilled water. The PCR reaction was 95 °C for 2 min, followed by 35 cycles of 95 °C 30 s, 55 °C 30 s, and 72 °C 1 min, and the last step was extension of 5 min at 72 °C. The PCR products were analyzed on 1 % agarose gels.

Purification of native SfAMY

The 10 g digestive gland of *S. forskali* in liquid nitrogen was ground and 40 ml of 20 mM sodium phosphate buffer (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, pH7.0) was added. The mixed solution was centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was then transferred to a new tube and stored at 4 °C until used. The SfAMY enzyme was purified by an affinity chromatography using self-couple β-cyclodextrin Sepharose 6B as affinity matrix. The 10 ml of the crude extract was loaded in the column (1 × 5 cm.) and washed with 100 ml of 20 mM sodium phosphate buffer, pH 7.0. The SfAMY enzyme was eluted by 10 mg/ml β-cyclodextrin in 20 mM sodium phosphate buffer, pH 7.0. The protein concentration was measured using the Bradford method and the purified SfAMY was stored at −80 °C [25].

Effect of temperature on SfAMY activity

The temperature effect on the activity of purified SfAMY was determined by incubating 56 μg of the purified enzyme with 250 μl of 1 % starch solution in phosphate buffer (20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 20 mM NaCl and 0.2 mM $CaCl_2$, pH7.0) for 5 min at different temperatures, ranging from 5 to 80 °C. The reaction was terminated by adding 250 μl of 3,5-dinitrosalicylic acid reagent and incubating in boiling water for 15 min. The solutions were cooled and diluted with 2 ml of distilled water. The absorbance of each mixture solution was measured by a spectrophotometer at 540 nm.

Effect of pH on amylase activity

To determine the effect of pH on SfAMY activity, 56 μg of the purified protein was mixed with different solutions of 250 μl of 1 % starch dissolved in the sodium phosphate buffer, each having different pH values ranging from 3 to12. The mixtures were then incubated at 40 °C for 5 min. To stop the reaction, 250 μl of 3,5-dinitrosalicylic acid reagent was added and then the reaction was boiled in a water bath for 15 min. After cooling, each solution was mixed with 2 ml of distilled water and the absorbance of the mixture solution was measured by spectrophotometer at 540 nm.

SfAMY activity assay by zymogram

Native polyacrylamide gel electrophoresis (native PAGE) was modified from the method of Laemmli [26]. Briefly, the purified enzyme was run at 120 V for 1 h on 12.5 % native polyacrylamide gel mixed with 1 % soluble starch in the sodium phosphate buffer pH 7.0. Then, the gel was incubated in the same buffer for 1 h. After washed with distilled water, the gel was stained with 10 mM iodine in 14 mM potassium iodide for 5 min. The excess iodine was removed by rinsing the gel with distilled water. The gel was then soaked in 1 % acetic acid for visualizing the α-amylase activity band. The gel was scanned using a Sony scanner.

Molecular weight determination

The molecular weight of the purified native SfAMY enzyme was determined using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme was run in a 12.5 % polyacrylamide gel and stained with Coomassie Brilliant Blue R-250. The gel was scanned using a Sony scanner.

Expression vector construction

In the nucleotide sequence encoding the mature SfAMY, the region from the 18th to 520th residue of the deduced sequence was amplified using cDNA as a template and a primer set, AMY-F and AMY-R (Table 1), under the following conditions: 94 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for

30 s, extending at 72 °C for 1 min and the final extending was carried out at 72 °C for 5 min. The amplified DNA was cloned into pGEM^{T} Easy plasmid vector and sent for sequencing at Macrogen, Korea. The plasmid was cut with two restriction enzymes, XbaI and NdeI. The obtained DNA fragment was ligated into the expression vector, pET 3a, to get a recombinant vector. The recombinant vector was transformed into competent cells, *E. coli* BL21 (DE3).

Extraction and purification of recombinant protein

E. coli BL21 (DE3) harboring the recombinant pET 3a plasmid was cultured and induced by isopropyl-β-thiogalactopyranoside (IPTG) to over-express the mature SfAMY. Briefly, 10 ml of the *E. coli* BL21 (DE3) starter culture was inoculated into 1 l 2X YT broth with 1 ml of 100 mg/ml ampicillin and 10 mM sucrose (0.2 % final concentration). The culture was shaken at 250 rpm at 37 °C until the optical density of *E. coli* cells reached the value of OD1, measured at 600 nm, and then the media was removed. The precipitated cells were suspended again with 1 l 2X YT broth and then 1 ml 0.4 M IPTG was added. After incubating for 4 h at 16 °C, the cultured cells were harvested by centrifuging at 4,000*g* for 30 min at 4 °C. The supernatant was removed and the cultured cells were frozen at −80 °C overnight. The frozen cells were thawed with 10 ml phosphate buffer and the cells were broken using a sonicator. The sonicated cell solution was centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was transferred to a new tube and the protein was then purified by an affinity chromatography using self-couple β-cyclodextrin Sepharose 6B as affinity matrix according to the method described in the purification of native SfAMY.

Molecular weight and activity assay of recombinant protein

The purified SfAMY recombinant protein was run on SDS-PAGE to estimate its molecular weight and was also run on a native PAGE to assay its activity. The optimal temperature and pH of the recombinant protein were assayed using the same methods described above.

Results

Nucleotide and deduced amino acid sequence of SfAMY

The full-length *α-amylase* cDNA of Indian rock oyster *S. forskali* was identified and submitted to GenBank (Accession No. KF478914). The *SfAmy* cDNA consisted of 1,689 bp and contained a 1,563-bp open reading frame (ORF). The 5′ untranslated region was 19 bp long followed by the start codon, ATG, at nucleotide position 20–22. The termination codon, TAG, was at position 1,561–1,563 followed by a 107-bp 3′ untranslated region containing a polyadenylation site (AATAAA) at position 1,623–1,628 (Fig. 1). The 1,563-bp ORF of *SfAmy* encoded 520 amino acid residues (Fig. 1). The deduced protein contained a putative 17 amino acid signal peptide predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The signal peptide would be cleaved off, yielding a 503 amino acid mature protein (Fig. 1). The predicted molecular mass and estimated isoelectric point (p*I*) of the mature SfAMY were 55.948 kDa and 6.45, respectively, as calculated using the compute pI/Mw tool of ExPASy web.

Comparison of amino acid sequence of SfAMY with other enzymes

The deduced amino acid sequence of SfAMY was aligned with those of other animal AMYs available in the NCBI database to identify identity percentage and conserved regions using ClustalW version 2.0.10. Among these AMYs, eleven were mollusk AMYs (Table 2). The amino acid sequences of mollusk AMYs could be divided into two groups based on the number of amino acids. One group had a short sequence of 509–523 amino acid residues, and the other group had long amino acid sequences ranging between 694 and 699 amino acid residues. The members of the first group were the AMYs of *S. forskali* (SfAMY), *Pinctada maxima* (PmAMY), *P. penguin* (PpAMY), *Pinctada fucata* (PfAMY), *Spondylus violaceus* (SvAMY), *C. gigas* (CgAMY A and CgAMY B), *H. discus discus* (HddAMY 1), and *H. discus hannai* (HdhAMY 58). The latter group consisted of three AMYs, *C. fluminae* AMY (CfAMY), HddAMY2, and HdhAMY82. The comparison of the amino acid sequences within the first group showed that the deduced sequence of SfAMY had 70–88 % identity with those of the bivalves and 63 % identity to those of the gastropods, while the percent identities between SfAMY and the AMYs of the latter group were less than 50 % (Table 2).

The multiple alignment also revealed that SfAMY contained several conserved motifs which were commonly found in animal AMYs such as active sites, calcium binding sites, chloride binding sites, substrate recognition sites, and cysteine residues. In the deduced SfAMY protein, two aspartic acid residues (D215 and D316) and a glutamic acid residue (E250) were present in the active sites for the catalytic activity. Four conserved histidine residues involved in substrate recognition were present at His120, His219, His315 and His340. Four amino acid residues, asparagine (N119), arginine (R171), aspartic

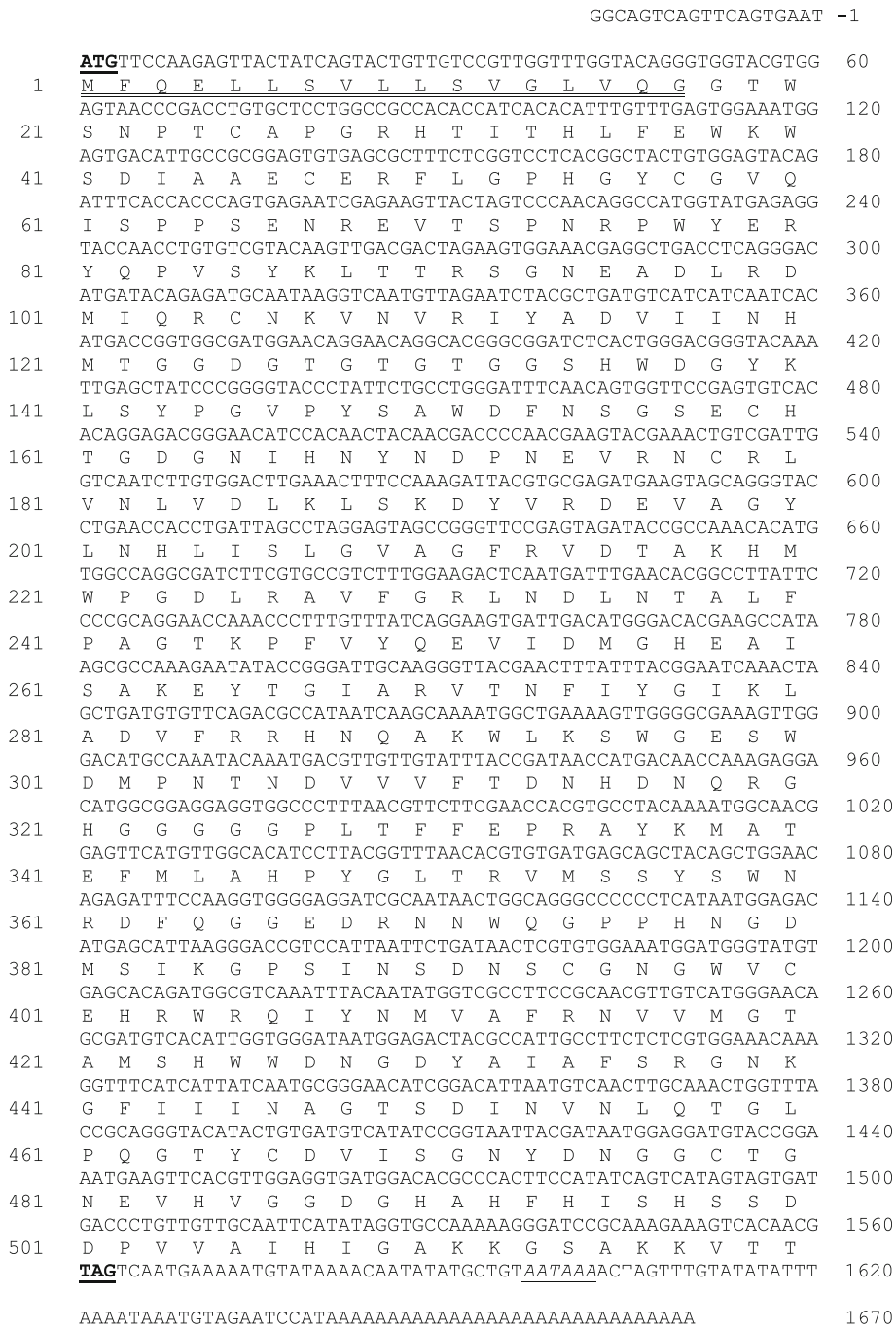


Fig. 1 Nucleotide and deduced amino acid sequences of *Saccostrea forskali* α-*amylase* gene. The *double line* in the deduced amino acid sequence indicates the putative signal peptide. The translation start site and the stop codon are in *bold* and *underlined*. The consensus polyadenylated signal sites are in *italics* and *underlined*. Nucleotides and deduced amino acids are numbered on the *right* and *left* hand sides, respectively

(D185), and histidine (H219) formed a putative calcium binding site. Moreover, three amino acid residues, arginine (R213), asparagine (N314), and glutamine (R352) involved in chloride binding required for full catalytic activity were found. Ten conserved cysteine residues were found at the amino acid positions 25, 47, 57, 105, 159, 178, 394, 400, 466, and 478 (Fig. 2), while the nine conserved motifs found in all animal AMY proteins were also present in the deduced SfAMY protein. The relatively conserved motifs 'WWERYQPVSYKL', 'GHGG', 'WTCEHRW' and 'DPXXAIH' of SfAMY and other mollusk AMYs are discussed later. These motifs were unique to all animal AMYs (Fig. 2).

Phylogenetic analysis for α-amylases

A phylogenetic tree of AMYs was constructed with the deduced amino acid sequences of all mollusk AMYs and some other animal AMYs available from the NCBI database using the maximum likelihood method. The AMY of the bacterium *Streptococcus equinus* was used as an out group. The obtained tree showed that animal

Table 2 Comparison of the mollusk AMY deduced amino acid sequences available in the NCBI database with *Saccostrea forskali* AMY deduced protein

Organism	Species	AMY name	Isoforms	Length (amino acids)	Identity	No. of cysteine residues	Accession number
Bivalve	*Saccostrea forskali*	SfAMY	1	520		10	KF478914
Bivalve	*Pinctada maxima*	PmAMY	1	518	77	11	AEI58897
Bivalve	*Pteria penguin*	PpAMY	1	523	78	10	AEI58894
Bivalve	*Pinctada fucata*	PfAMY	1	522	76	10	AGN55420
Bivalve	*Crassostrea gigas*	CgAMY A	2	520	88	10	AAL37183
		CgAMY B		519	83	10	AAL37207
Bivalve	*Spondylus violaceus*	SvAMY	1	509	70	11	AFE48186
Bivalve	*Corbicula fluminae*	CfAMY	1	699	45	17	AAO17927
Gastropod	*Haliotis discus discus*	HddAMY 1	2	511	63	10	ABO26610
		HddAMY 2		694	46	18	ABO26611
Gastropod	*Haliotis discus hannai*	HdhAMY 58	2	511	63	10	BAM74656
		HdhAMY 82		694	46	18	BAM74657

AMYs were divided into two major groups. One group comprised all animal AMYs with short sequences, while the other group contained the three mollusk AMYs with long sequences (L, long sequence) (Fig. 3). The first group was also separated into three subgroups, mollusk S (S, short sequence) AMYs including SfAMY, vertebrate AMYs and insect AMYs.

SfAmy expression analysis by RT-PCR

SfAmy expression was studied using the RT-PCR technique. As expected, the results showed that expression was highest in the digestive gland, moderate in labial palps, low in gill and mantle, and none in adductor muscle. The ubiquitously expressed *β-actin* gene was found at the same level in all tissues examined (Fig. 4).

Molecular mass and enzyme activity of the native SfAMY enzyme

The molecular mass of SfAMY was identified using SDS-PAGE. The result showed that the molecular mass of the purified native SfAMY enzyme was approximately 56 kDa, which was consistent with the molecular mass calculated from the deduced amino acid sequence of the *SfAmy* cDNA (Fig. 5a). Moreover, the protein showed only one band on a native PAGE indicating that the enzyme had only one isoform (Fig. 5b). The specific activity of the purified native SfAMY, at 6.34-fold purification, was 187.42 U/mg. The optimal temperature and pH of the native enzyme were 40 °C and 6.0, respectively (Table 3).

SfAMY recombinant protein

The cDNA fragment for producing the recombinant SfAMY as a mature form was amplified and cloned into the pGEM^{T} Easy plasmid vector. The cDNA fragment was then subcloned into the expression vector, pET 3a. The recombinant SfAMY protein was overexpressed in *E. coli* cells, BL21 (DE3) (Fig. 6a). The purified recombinant enzyme also showed the same size, ~56 kDa, as the native one (Fig. 6b). The crude and purified recombinant enzymes were then tested for their activities on the native PAGE. Both crude and purified recombinant enzymes could act on a 1 % starch substrate (Fig. 6c). The specific activity of purified recombinant SfAMY was 46 U/mg which was 4 times higher than the crude recombinant extract (11.8 U/mg). Moreover, its optimal temperature and pH were 40 °C and 5.0, respectively (Table 3).

Discussion

Analysis of full-length *SfAmy* cDNA and deduced protein

In this study, the *α-amylase* gene encoding the α-amylase enzyme of Indian rock oyster, *Saccostrea forskali*, was identified. The full-length *SfAmy* cDNA sequence consisted of 1,689 bp containing a 1,563-bp ORF. The *SfAmy* cDNA sequence had a short 5′UTR, of 19 bp, similar to that of most mollusk *Amy* cDNA genes such as 18 bp in *PpAmy*, 17 bp in *PfAmy*, 18 bp in *HddAmy1*, 7 bp in *HddAmy2*, 21 bp in *HdhAmy58* and 12 bp in *HdhAmy 82*. The *SfAmy* ORF encoded the deduced 520 amino acid deduced protein, whose length was within the length of most animal AMYs. The deduced length of eleven mollusk AMYs available in

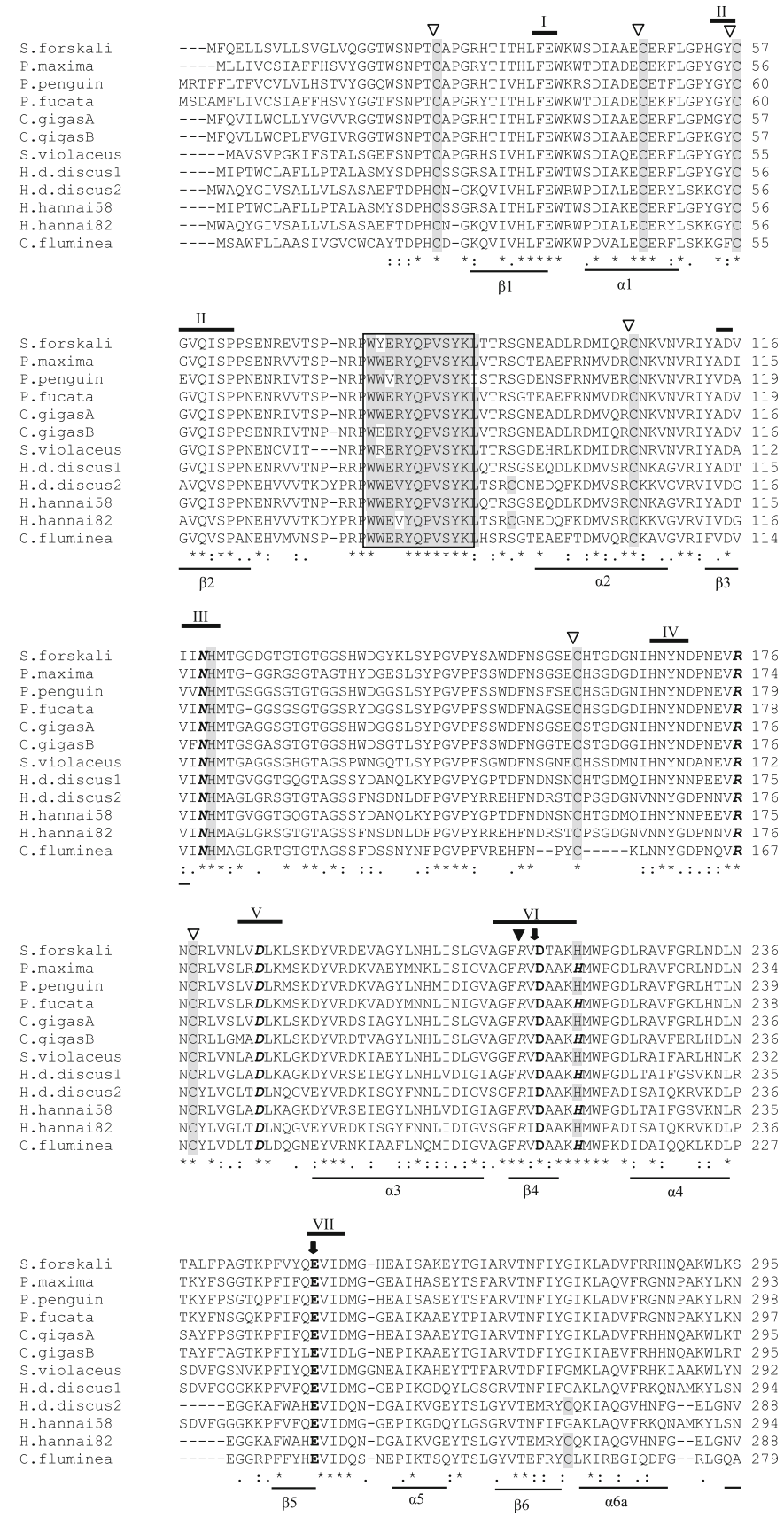
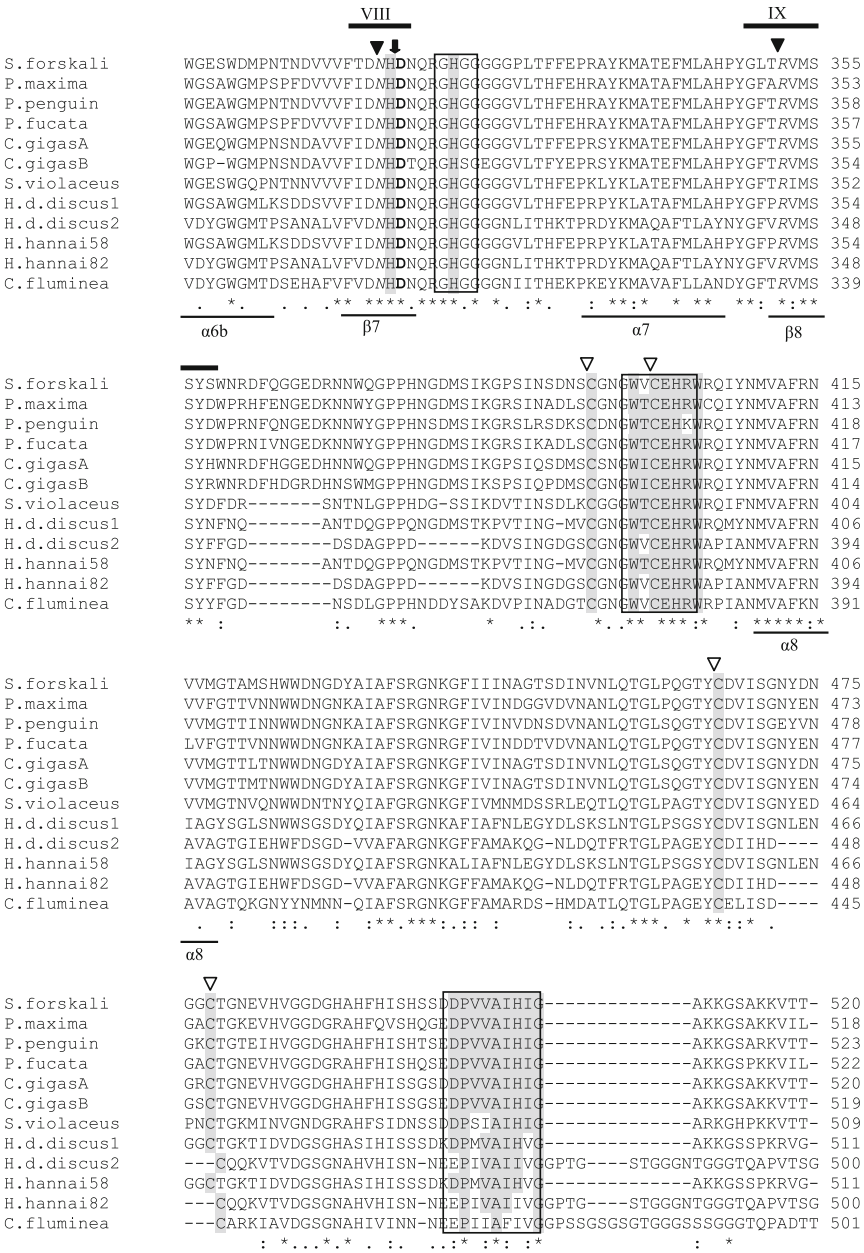


Fig. 2 Multiple alignment of *Saccostrea forskali* and other mollusk α-amylase amino acid sequences. The nine conserved regions are indicated with *thick lines with Roman numerals above* the amino acid sequences. The (β/α) 8-barrel structure elements are under the amino acid sequences with *thin lines*. Active site residues (D215, E250 and D316) are *bold* and marked with a *down arrow* over the columns. Chloride binding residues (R213, N314 and R352) are *italic* and marked with a *filled down triangle* over the columns. Calcium binding residues (N119, R171, D185 and H219) are italic. Substrate recognition residues (H120, 219, 315, and 340) are shaded in *gray*. Ten conserved cysteine residues are shaded in *gray* and marked with an *open down triangle* over the columns. *Boxes* indicate interesting motifs: see text in discussion. All amino acid sequences were obtained from the NCBI database

Fig. 2 continued



the NCBI database ranged between 509–699 amino acid residues. Of these eleven AMYs, eight mollusk AMYs were about 500 amino acid residues in length while the remaining three mollusk AMYs consisted of 694–699 amino acid residues. These three long mollusk AMYs had a stretch of about 500 amino acid residues at their N-terminus that were similar to the amino acid sequences of all other mollusk AMYs, while a domain of approximately 190 residues did extend at the C-terminus. This extension sequence was called the C-terminal ancillary domain [14, 18]. The calculated molecular mass of the mature SfAMY protein was 55.948 kDa. This molecular mass was confirmed by the purified native SfAMY run on a SDS-PAGE. Generally, the molecular mass of animal AMYs were within the range of 45–67 kDa [11, 27].

The isoelectric point (p*I*) of SfAMY was 6.36 and was similar to those of bivalve mollusks such as PmAMY (7.62), PpAMY (7.35), CgAMYA (6.65), CgAMYB (6.62), and SvAMY (7.31), while the p*I* values of marine gastropod AMYs were higher, for example HddAMY 1 (8.3) [13] and HdhAMY 58 (8.18) [14]. On the other hand, the AMYs with ancillary domains have low p*I* values <6.0. The differences of p*I* values suggest that the p*I* values may reflect the phylogenetic relationship, food resources, and the origin of the enzymes [28]. In insects, the difference in p*I* values may be a response to food resources, since those

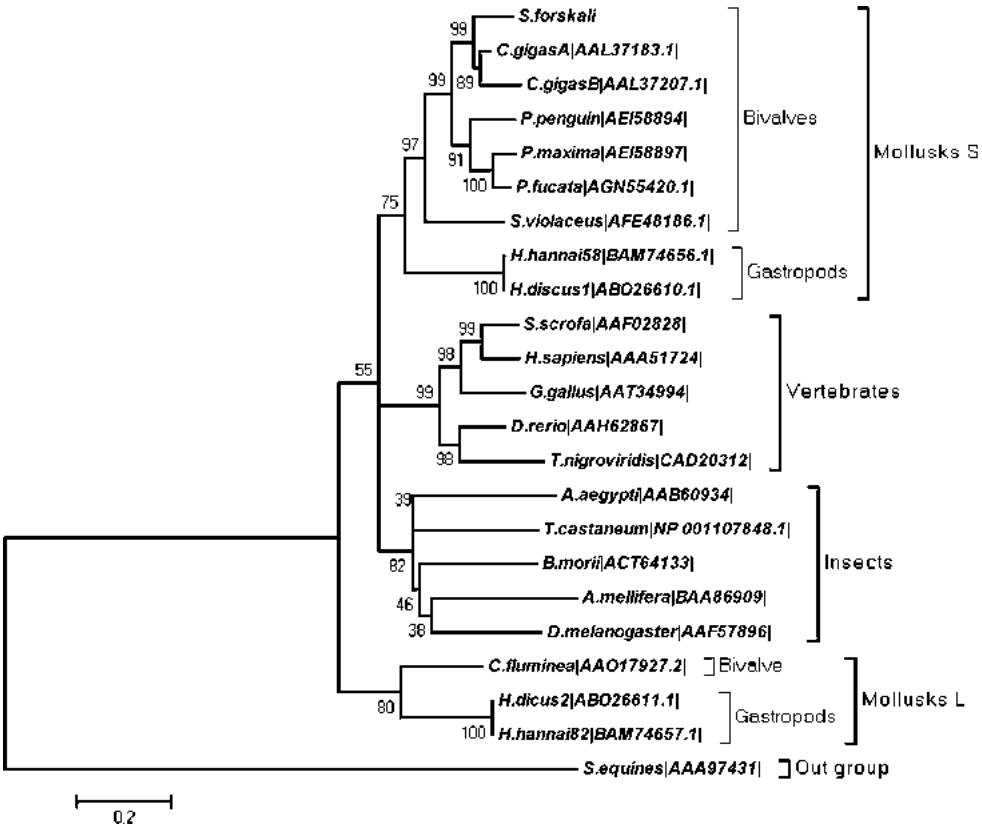


Fig. 3 Phylogenetic tree constructed by MEGA 5.05 using the deduced amino acid sequences of α-amylase from *Saccostrea forskali* and some other animals. The numbers at the nodes were the percentage of 1,000 bootstrap re-samples

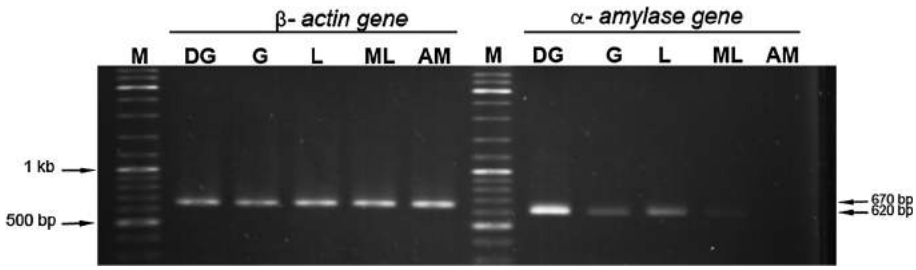


Fig. 4 Gel analysis of tissue-specific expression of *Saccostrea forskali α-amylase* gene using RT-PCR. *β-actin* gene (670 bp bands) was used as an internal control. *M* markers, *DG* digestive gland, *G* gill, *L* labia palps, *ML* mantle, and *AM* adductor muscle

of AMYs from insects living on dry foods such as *Sitophilus oryzae*, *Rhyzopertha dominica*, and *Anagasta kuehniella*, ranged from 3.7 to 4.2, while p*I* values of 6.0–6.3 are observed in the AMYs of insects feeding on soft plants such as *Bemisia tabaci*, *Pheropsophus aequinoctialis* and *Erinnyis ello*. Also, the enzymes in different organs such as gut and salivary glands have different *p*I values [28]. Therefore, the difference in *p*I values among mollusk AMYs also suggests a functional variation among molluskan AMYs.

Multiple alignment of the deduced amino acid sequence of SfAMY with that of some other animals reveals that SfAMY had all the conserved motifs that were commonly found in all animal AMYs such as the active sites, calcium biding sites, chloride binding sites, and substrate recognition sites [15]. All animal AMYs and three extremophillic Gram-negative bacteria AMYs are chloride-dependent enzymes that require chloride for full activity [15, 17, 21]. The three amino acid residues involved in the chloride binding of most animal AMYs, including SfAMY, were two arginine (R) residues and an asparagine (N) residue [29], while in the AMYs of lepidopteran insects, one arginine was replaced by glutamine (Q) [12, 30, 31].

Another interesting aspect of the conserved sequences of animal AMYs is the number of cysteine residues. All animal AMYs contain eight conserved cysteine residues that form four disulfide bridges [16]. However, some animal AMYs have additional cysteine residues, for example 12 residues in the pig *Sus scrofa* [8], and silkworm *Bombyx mori* [31]; 10 residues in the shrimp *Penaeus vannamei* [6], and mosquito *Aedes aegypti* [32].

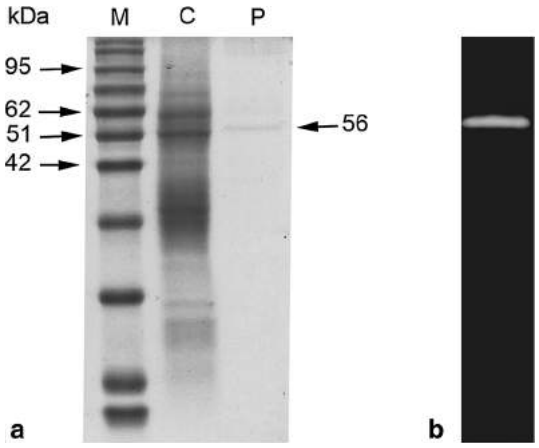


Fig. 5 Electrophoretic analysis of native SfAMY. **a** SDS-PAGE on 12 % polyacrylamide gel of crude and purified native enzymes stained with Coomassie Brilliant Blue R-250. *M* marker (Genedirex), *C* crude enzyme and *P* purified enzyme. **b** A zymogram of the crude native enzyme in native PAGE using 1 % soluble starch as substrate and stained with KI-I_2

In this study, SfAMY contains 10 cysteine residues that were conserved in all mollusk AMYs. Out of 10 cysteine residues, those residues in 8 positions were found in all animal AMYs. The other two residues (Cys25 and 57) were found in the N terminal region of SfAMY and other mollusk AMYs but were not found in other animal AMYs. These two cysteines may form another disulfide bridge, the fifth disulfide bridge, that may help to reduce degradation from the N terminus of the enzyme [21]. In addition, the AMYs of some other animals such as mammals, insects and crustaceans, contained an additional disulfide bridge, but this bridge was formed by additional cysteine residues in different positions, depending on species [21]. The AMYs of some mollusks have more than 10 cysteine residues, such as 11 in *P. maxima* and *S. violaceus*. In the three long mollusk AMYs, the number of cysteine residues in HddAMY2 and HdhAMY82 was 18 while it was 17 in CfAMY. These 17/18 cysteine residues were the ten basic conserved cysteines found in all mollusk AMYs, six conserved cysteines found in the ancillary domain, and the remaining one/two cysteines found in domain A.

Moreover, animal AMYs had some similar motifs that were not shared with other organisms [12]. The first motif was the 12 amino acid motif 'WYERYQPVSYKL', between the β2 and α2 of SfAMY. Some amino acids of this motif were modified. Out of twelve mollusk AMYs, seven AMYs possessed the motif 'WWERYQPVSYKL'. The modifications occurred in the second amino acid position (Y, W, E, R), the third (E, V), the fourth (R, V)

Table 3 Summary of the purification of native and recombinant α-amylase from *Saccostrea forskali*

Type	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude native SfAMY	202	68.25	29.53	100	1
Purified native SfAMY	8	0.42	187.42	4	6.34
Crude recombinant SfAMY	464	393.2	11.8	100	1
Purified recombinant SfAMY	7.43	1.57	46	1.6	4

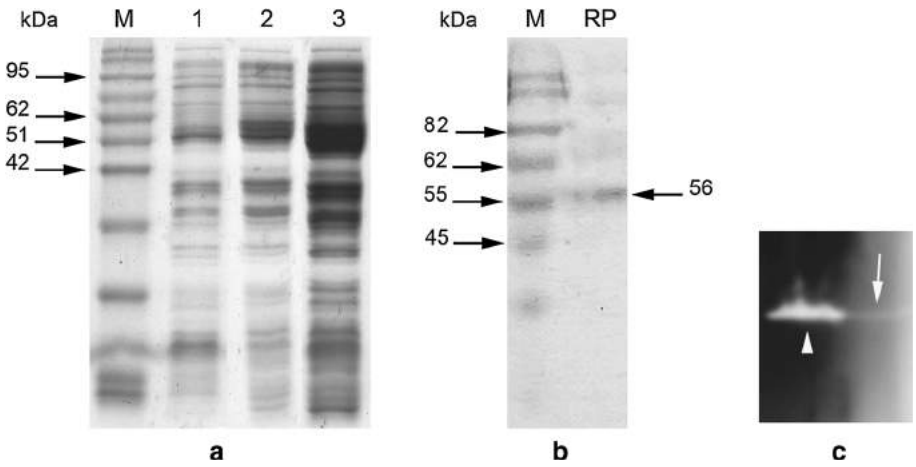


Fig. 6 SDS-PAGEs of recombinant SfAMY expressed in *E. coli* BL21. **a** The expression of the enzyme: *M* protein marker in kDa (Genedirex), *1* total cellular extract without IPTG induction after incubation at 25 °C for 4 h, *2* and *3* total cellular extract after IPTG induction at 25 °C for 2 and 4 h, respectively. **b** The purified recombinant protein: *M* marker (Vivantis) and *RP* recombinant protein. **c** Zymogram PAGE of recombinant SfAMY activities. An *arrowhead* and an *arrow* point to the activity of crude and purified enzymes, respectively

and the twelfth (L, I). The second was the GHGG motif which was in the flexible loop. It has been suggested that this motif could be involved in a trap-release mechanism for hydrolyzing substrates [33, 34]. All mollusk AMYs contained the GHGG motif except CgAMY B which instead possessed the GHSG motif, while the GHGA motif was often found in other animals. The third motif was 'WTCEHRW', located close to the α8. Out of seven amino acids, the second and the sixth amino acids were modified. In mollusk AMYs, the amino acids present in the second position were the non-polar amino acids V, T, and I. In SfAMY and in the three AMYs with long sequences, the second amino acid of this motif was valine (V). In the sixth position, the arginine (R) was present in all mollusk AMYs, except PpAMY which possessed a lysine (K). Both arginine and lysine are basic amino acids.

Another interesting motif was the DPX_1X_2AIH sequence at the C-terminal tail of SfAMY. The first two amino acids, D and P, were often present in most mollusk and vertebrate AMYs. However, the DP was replaced with DM in lepidopteran AMYs, and with DG in *Drosophila* [30], whereas the mollusk AMYs with the ancillary tail possessed EP. In addition, the two amino acids following the DP were often hydrophobic such as valine (V), isoleucine (I), methionine (M), or serine (S). In SfAMY, these two amino acids were valines (VV). The triple amino acid sequence, AIH, was present only in mollusk AMYs with short sequences. Moreover, in most animals, the amino acids following the AIH are hydrophobic. In all mollusk AMYs, the two amino acids (IG/VG) following the AIH triplet are hydrophobic. However, some insects such as *Helicoverpa armigera* and *Spodoptera frugiperda*, have hydrophilic amino acids in this position instead [30].

Among mollusk AMYs, the identity of SfAMY to bivalve and gastropod AMYs with short sequences was 70–88 and 63 % respectively, whereas the identity between SfAMY and the other three long mollusk AMYs with ancillary domains was 45–46 %, which was lower than the 50 % identity between SfAMY and the AMYs of other animal groups such as humans, pigs, fruit flies, and mosquitos. Moreover, even if the ancillary domains in their C-terminuses were excluded from analysis, the identity of SfAMY to the three long mollusk AMYs was still lower than 50 %. However, all mollusk AMYs were members of the GHF-13 α-amylase [14].

A phylogenetic tree was constructed using the deduced amino acid sequences of these mollusk AMYs and some other known animal AMYs. The tree showed that these animal AMYs could be divided into two major groups. One contained all AMYs whose length was approximately 500 amino acids, and the other one was composed of the three long AMYs. The phylogenetic tree also presented the same topology even though the ancillary domains in the C-terminus were excluded from the analysis.

Enzyme analysis

In mollusks, the existence of one to three isoforms of AMYs has been reported. For example, two isoforms of AMYs were found *C. gigas* [11], *H. discus hannai* [14], and *Mytilus galloprovincialis* [35], one isoform of AMY was found in *Haliotis sieboldii* [36], and three isoforms were reported in *Meretrix lusoria* [37]. In this study, the existence of only one isoform was confirmed by the zymogram and SDS PAGE of the purified native SfAMY. Multiple AMY isoforms had been found in many animals, particularly in insects, for example two isoforms in *Zabrotes subfasciatus* [38], five isoforms in *Morimus funerus* [39], and seven isoforms in *Cerambyx cerdo* L. [40]. In humans and rodents, the AMYs have two isoforms [41, 42]. It has been suggested that the multiple isoforms of several insect AMYs enhance their adaptability to different foods and enables them to overcome the inhibitor produced by food plants [43]. It has also been suggested that the occurrence of two AMY isoforms, CgAMY A andCg AMY B, in *C. gigas* made the oyster adaptable to food and environmental variations [44]. Therefore, the presence of only one isoform of AMY in *S. forskali* may be sufficient for digesting foods that may not contain any inhibiting substances.

The optimal temperatures of mollusk AMYs studied so far ranged from 30 to 50 °C [13, 14, 35–37]. In this study, the activity of native and recombinant SfAMYs showed the same optimal temperature of 40 °C, which was within this range. The optimal pH values of invertebrate and mammal AMYs had been reported to be near to neutrality [45]. The optimal pH of AMYs varied depending on species. Alkaline pH optima were reported for the AMYs of lepidopteran insects such as pH 9.8 in *Bombyx mori* [46], and pH 9.0 in *Naranga aenescens* [47]. Also, the optimum pH of penaeid shrimp AMYs are alkaline [48]. In some other organisms AMYs work well at acidic pH, such as pH 4.5–5.0 in *Sitophilus* sp. [49] and pH 5.5 in *Morimus funereus* [39]. All mollusk AMYs studied so far were active in neutral to slightly acid pH conditions (6.0 to 7.5) [13, 14, 35–37]. In this study, the native and recombinant SfAMYs showed optimal activity at pHs of 6.0 and 5.0, respectively, which is in the range of the optimum pH values for the activity of the other mollusk AMYs.

Acknowledgments We are grateful to Dr. Nora Fascestti for critical reading of manuscript. We thank the Higher Education Commission of Thailand and the Center of Agricultural Biotechnology, Kasetsart University, Kampaengsaen Campus, Nakon Pathom, for the grant to TT.

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