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Amino Acid Sequence of Amylase Type Alpha, MiAmy, from Ok-Rong Mango (*Mangifera indica* Linn. cv. Ok-Rong)

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Abstract: The 594-amino acid residue sequence of α-amylase, MiAmy, from the Ok-rong mango (*Mangifera indica* Linn. cv. Ok-rong), in the ripening stage, was determined through Reverse Transcription Polymerase Chain Reaction (RT-PCR). Sequence alignments and evolutionary tree analyses revealed its high similarity to plastid α-amylase from many other plants. The sequence was revealed to have four conserved regions with catalytic amino acid residues for the active site. It was classified as a member of α-amylase family 13 because it has an active Domain A, similar to a $(\beta/\alpha)_8$-barrel structure. Three-dimensional structural predictions revealed that this partial sequence completely covered all of necessary domains for amylase activity.

Keywords: α-Amylase, Ok-Rong Mango, Amylase Family 13, Amino Acid Sequence, *Mangifera indica*

Introduction

α Amylases are classified into amylase family-13 (EC 3.2.1.1) or GH-13 which hydrolyzes α-1, 4-D-glucosidic bonds. These proteins are found in diverse organisms and are still being characterized. α Amylases can be utilized for diverse applications, such as in the detergent, textile, paper, sugar and ethanol industries. For each application, it is important to understand the enzyme's specificity and function. Recently, a large number of α amylase proteins have been discovered in sources such as bacteria, fungi, cereals, mammal and higher plants including fruits (Torgerson *et al*., 1979; Kondo *et al*., 1980; Robyt and French, 1970; MacGregor and MacGregor, 1985; Stanley *et al*., 2002; SWISS-PROT, 2000; GenBank, 1982). These newly discovered enzymes vary in their structures and specificity.

Ok-rong mango (*Mangifera indica* Linn. cv. Ok-rong) is a plant indigenous to Thailand (local name; Mamuang Ok-rong). It is a large green tree that grows up to 20 m tall. Mangos thrive in both the subtropics and the tropics and are one of the most popular fruits both in ripe and green stages. The ripening process involves changes to several biochemical compounds which in turn cause changes in color, flavor, texture and taste. For example, in regard to flavor, soluble sugars, account for mango sweetness and accumulate through carbon supplied during both photosynthesis and starch degradation, which occur during ripening. During fruit development, starch accumulates up to 8% in the fresh pulp weight, but a low amount of soluble sugars is detected. However, during ripening, accumulated starch is rapidly converted into soluble sugars, which that can reach as high as 10% of the fresh pulp weight (Peroni Goncalves *et al*., 2008).

In previous studies, the amylase activity during ripening of Ok-rong mangos was significantly higher than in 30 other tested fruits (personal communication). Therefore, it is possible to discover an isoform of α-amylase that has a high specific activity, many favorable properties and a high potential for development for industrial applications.

Materials and Methods

Plant Materials

Ok-rong mangos (*Mangifera indica*) in the ripening stage of were collected from Srisaket Province in

northeastern Thailand. Pulp was ground under liquid nitrogen and stored at -80°C before use.

Amino Acid Sequence Determination

Ripening Ok-rong mango tissues were disrupted by the addition of liquid nitrogen and subsequent homogenization. Total RNA was extracted using TRIzol® reagent. Then, cDNA sample were synthesized using the cDNA synthesis kit (ThermoScript™). For α amylase gene amplification, primers (Table 1) were designed from the α amylase sequences from other plants, *Arabidopsis* thaliana; AY065233, *Malus domestica*; AAF63939 and *Citrus sinensis*; XP_006483229 (Genbank). Next, specific primers were designed from our α amylase sequence obtained above. For PCR reactions, pre-denaturation was carried out at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min. A final extension step at 72°C was conducted for 10 min. The amplicons were purified and ligated into the pGEM-T Easy vector (Promega, USA). DNA plasmids were purified and sequenced (1st Base Company, Malaysia).

The 3′ RACE system was carried out according to the kit's instruction manual (Invitrogen, USA) using the AUAP universal primer as a reverse primer. The PCR conditions were to mix sample in the green PCR master mix kit (Fermentas, Singapore) and thermocycle at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and final extension at 72°C for 10 min. Then, the amplicons were purified and ligated into the pGEM-T Easy vector (Promega, USA). DNA plasmid were purified and sequenced.

Bioinformatics

Nucleotides sequences were blasted using local basic alignment search BLAST® tools (http://blast.ncbi.nlm.nih.gov) and converted to amino acid sequences using ExPASy translation tool (http://web.expasy.org/translate). Amino acid sequence alignments were performed using the CLUSTALW program (http://ebi.ac.uk/Tools/msa/clustalw2/) using sequences from several publications and the Genbank DNA sequence database (Table 2) (Larkin *et al*., 2007). Phylogenetic tree and evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA6; http://megasoftware.net/) (Tamura *et al*., 2013). Three-dimensional structural predictions were launched using the SPDBV Swiss model program (http://spdbv.vital-it.ch/) (Guex and Peitsch, 1997) and the catalytic region domain was confirmed using the InterProScan Swiss model program (http://swissmodel.expasy.org/work-space/) (Zdobnov and Apweiler, 2001).

Table 1. Primers for determination of α amylase from Ok-rong mango (*M. indica*) nucleotide sequences

Primer pairs	Forward primer sequence	Reverse primer sequence	Expected size (bp)
FM1RM1	5′-GGAGGCTTGA TTTCGTTAGAG-3′	5′-GCGTAGACGT CTCTTTCTGCC-3′	511
RM2	5′-CAGGGTCATT	5′-GGCCACGCG	311
AUAP	GGAGGTTTC-3′	TCGACTAGTAC-3′	
FM2RM3	5′-GATGCTTCT TAAAACCACC G-3′	5′-GGCATCTTG ATTGTGGTCC-3′	435
FM3RM4	5′-GTTGTTCACTG GGGAGTTTG-3′	5′-GTGCCTCAG TTTCCAAAACA AC-3′	500
FM3RM3	5′-GTTGTTCACTG GGGAGTTTG-3′	5′-GGCATCTTG ATTGTGGTCC-3′	1300
FM4RM3	5′-CTTCCAGGGA AATGGATCTC-3′	5′-GGCATCTTG ATTGTGGTCC-3′	1500

Table 2. α Amylases included in the present study

Plant species	Common name	Accession number	Abbreviation	α Amylase group
Malus domestica	Apple	AAF63239	MdAmyF1	Secretory
Solanum tuberosum	Potato	M79328	StAmyF1	Secretory
Oryza sativa	Rice	P17654	OsAmyF1	Secretory
Hordeum vulgare	Barley	P00693	HvAmyF1	Secretory
Musa acuminata	Banana	AF533648	MaAmyF1	Secretory
Triticum aestivum	Wheat	P08117	TaAmyF1	Secretory
Zea mays	Maize	AAA50161	ZmAmyF1	Secretory
Actinidia chinensis	Kiwi	AAX33233	AcAmyF3	Plastid
Morus notabilis	Mulberry	XP_010096344	MnAmyF3	Plastid
Malus domestica	Apple	NP_001281020	MdAmyF3	Plastid
Citrus sinensis	Orange	XP_006483229	CsAmyF3	Plastid
Arabidopsis thaliana	Arabidopsis	NP_564977	AtAmyF3	Plastid
Glycine soja	Wild soybean	KHN13489	GsAmyF3	Plastid
Ricinus communis	Castor bean	XP_002520134	RcAmyF3	Plastid
Durio zibethinus Murr. cv.Mon Thong	Durian	KP164993	DzAmyF3	Plastid
Mangifera indica Linn cv.Ok-rong	Ok-rong mango	-	MiAmy	This study

Results

MiAmy Amino Acid Sequence Determination

Five nucleotide fragments were obtained from RT-PCR techniques using 5 pairs of primers. The 3′ end was determined using 3′ RACE. There were completely overlapped (Fig. 1). Six sequence fragments were found to be part of the α amylase sequence. The sequence was found to be 1782 base pairs of nucleotides and share 85, 83 and 79% identity with α amylase from durian (*D. zibethinus* cv. Mon thong), sweet orange (*C. sinensis*), mulberry (*M. notabilis*), respectively. The nucleotide sequence encoded 594 amino acid residues which shared 78 and 77% identity with chloroplastid α amylase 3, from *D. zibethinus* and *C. sinensis*, respectively. It was named MiAmy.

Multiple Alignment for Sequence Similarities

Well-described sequences from previous studies (Nakajima *et al*., 1986; Janecek, 1992) were inputs into the CLUSTALW program and used to generate amino acid sequence alignments for α-amylase. The large N-terminal domain of plastid α-amylase, amino acids 447-460, was removed. The pre-defined sequences contained four conserved and semi-conserved positions, including the active site, which allowed similarities to be identified for the same regions in the novel sequence. The alignments revealed high conservation between sequence with substitutions by similar amino acid residues when compared with those from other sources including plants, mammals and bacteria. The four conserved regions are region (I) DAVLNH, (II) GWRLDFVRG, (III) GEYWD and (IV) FIENHDT. From structural studies, region II and III are suggested to be specific for the α-1, 4 glucosidic bond for α amylase family 13. The study of their anomeric configuration revealed their activity at one end of a polysaccharide (MacGregor *et al*., 2001). The four highly conserved residues are the substrate binding site; H291 and the catalytic sites; D372, E397 and D481 presented in conserved region I, II, III and IV, respectively (Fig. 2).

Evolution Tree of α Amylase Ok-Rong Mango

Phylogenetic trees with unrooted distances were calculated based on the Neighbor-Joining method (Saitou and Nei, 1987) based on the sequence similarities alignment (Fig. 2). The MiAmy was grouped into clusters along with the plastid α amylase members (Fig. 3). The tree reflects data from previous studies, such as a difference between conserved amino acid residues in the conserved region, especially region four. For grouping of only plastid α amylases, MiAmy was branched nearest to DzAmyF3 (*D. zibethinus* cv. Mon Thong) and CsAmyF3 (*C. sinensis*). This evolutionary study corresponds to the high (78 and 77%) similarity of alignment confirming that MiAmy is an α amylase from plastids.

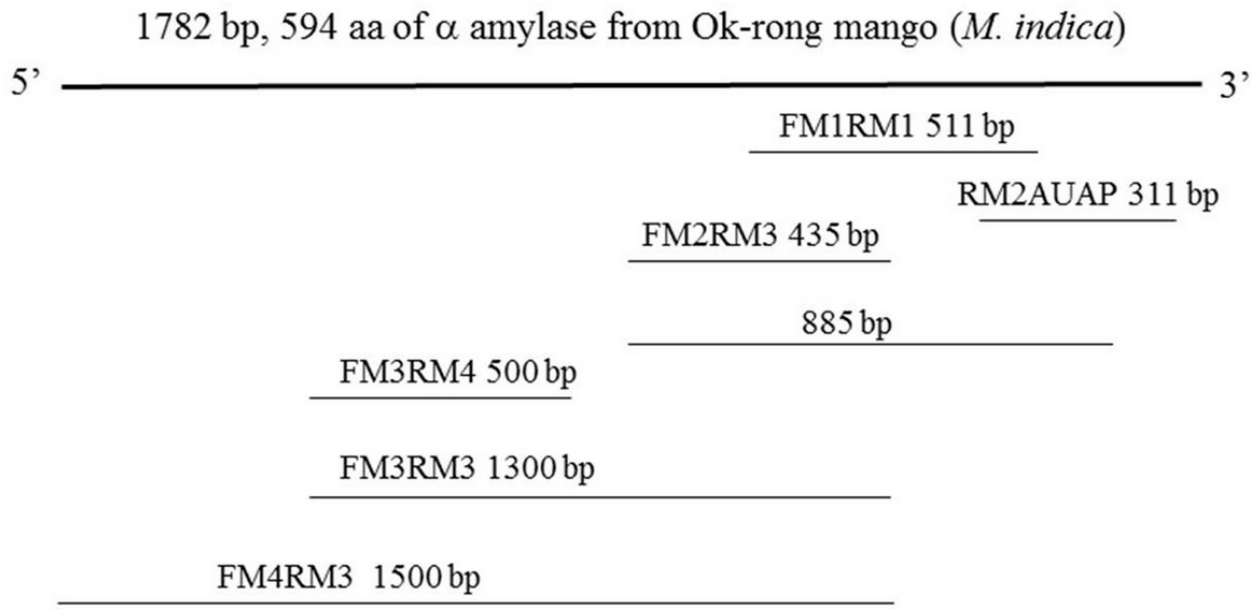
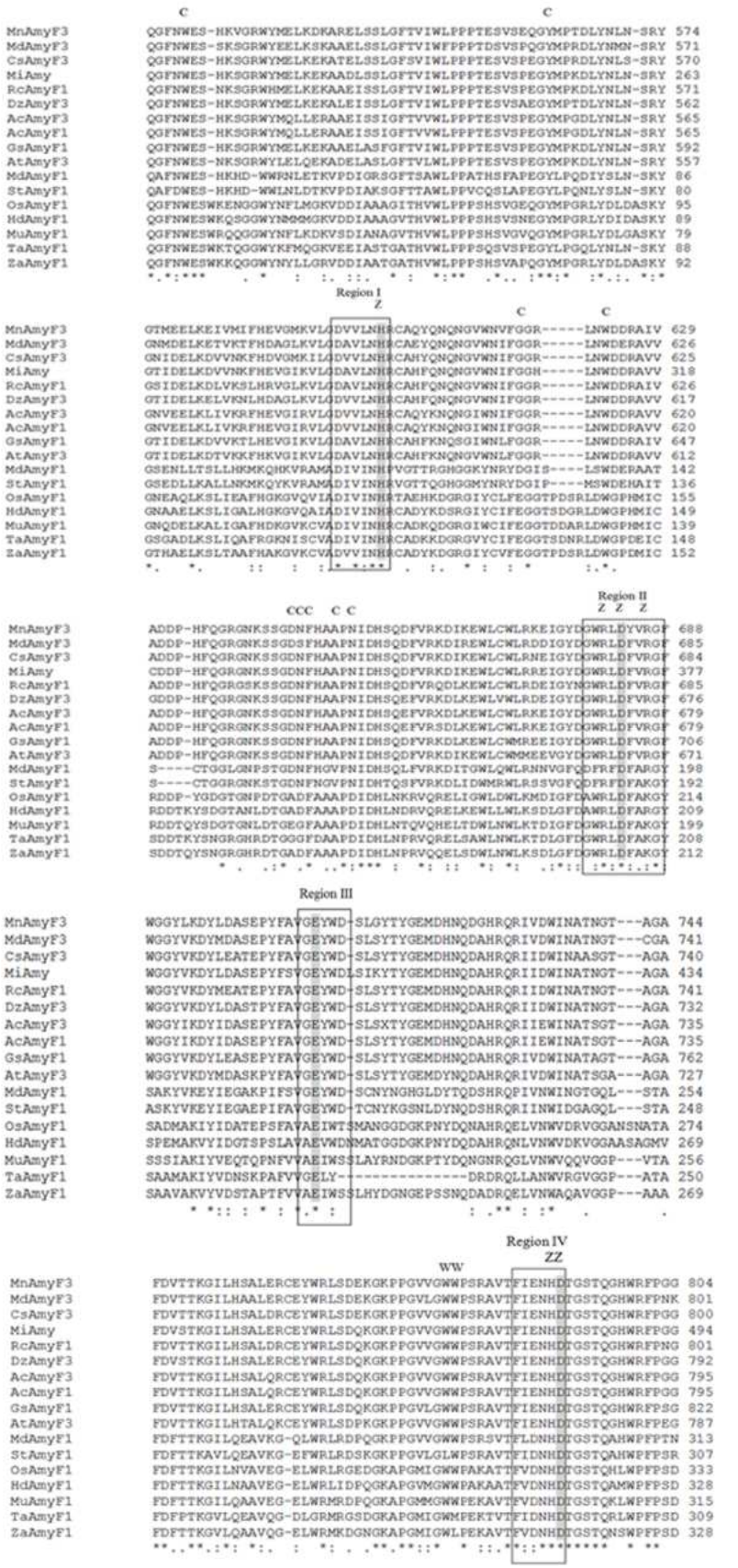


Fig. 1. Amino acid sequence strategies. The 594-amino acid sequence of α-amylase was deduced from overlapping a map of the α-amylase gene, determined by cDNA walking and 3′-RACE from Ok-rong mango (*Mangifera indica*)


Region I
Region II
Region III
Region IV
MnAmyF3
MdAmyF3
CsAmyF3
MiAmy
RcAmyF1
DzAmyF3
AcAmyF3
AcAmyF1
GsAmyF1
AtAmyF3
MdAmyF1
StAmyF1
OsAmyF1
HdAmyF1
MuAmyF1
TaAmyF1
ZaAmyF1

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MnAmyF3         KEIQGYAYILTHPGTPSVFYDHIFSH---YGSEIGSLISLRNRNKIHCRSRVQITKAERD 861
MdAmyF3         KEMQGYAYILTHPGTPTVFYDHIFSH---YQSEIAALISLRNRNKLNCRSRVKITKAERD 858
CsAmyF3         REMQGYAYILTHPGTPSVFYDHIFSH---YRQEIEALLSVRKRNKIHCRSRVEIVKAERD 857
MiAmy           KELQGYAYILTHPGTPAVFYDHIFSN---YRSEIEALISLRKRNKLHCRSIVQIAKAERD 551
RcAmyF1         KEMQGYAYILTHPGTPTVFYDHIFSH---YRSEIASLISLRKRNEIHCRSSVKITKAERD 858
DzAmyF3         KELQGYAYILTHPGTPAVFYDHISSH---YRSEIAALISLRNRNKIHCRSTVKIVKAERD 849
AcAmyF3         KEMQGYAYILTHPGTPAVFYDHAFHH---MRSEISALVSLRNRNKIHCRSTIQITKAERD 852
AcAmyF1         KEMQGYAYILTHPGTPAVFYDHAFHR---MRSEISALVSLRNRNKIHCRSTIQITKAERD 852
GsAmyF1         KEMQGYAYTLTHPGTPSVFYDHIFSH---YKTEIATLISIRKRNKIHCRSTLKICKAERD 879
AtAmyF3         KEMQGYAYILTHPGTPAVFFDHIFSD---YHSEIAALLSLRNRQKLHCRSEVNIDKSERD 844
MdAmyF1         HIMEGYTYILTHPGIPTVFYDHFYDWGDSIHDQIVKLIDIRKRQDIHSRSSITILEAQPN 373
StAmyF1         HVMEGYAYILTHPGIPSVFFDHFYEWDNSMHDQIVKLIAIRRNQGIHSRSSIRILEAQPN 367
OsAmyF1         KVMQGYAYILTHPGNPCIFYDHFFDWG--LKEEIERLVSIRNRQGIHPASELRIMEADSD 391
HdAmyF1         KVMQGYAYILTHPGIPCIFYDHFFNWG--FKDQIAALVAIRKRNGITATSALKILMHEGD 386
MuAmyF1         KVMQGYAYILTHPGVPSIFYDHMFDWG--LKEKITRLAKTRTRNRIHSGSSLNILASDAD 373
TaAmyF1         KVMQGYAYILTHPGIPCIFYDHVFDWK--LKQEITALATVRSRNGIHPGSTLDILKAEGD 367
ZaAmyF1         KVMQGYAYILTHPGTPCIFYDHVFDWN--LKQEISALSAVRSRNGIHPGSELNILAADGD 386
                : ::**:* ***** * :*:**          :*  *   * .: :   * : *   : :
                                        S
MnAmyF3         VYAAIIDEKVAVKIGPGHYEP-PSGPQRWSRAVEGRDYKVWEAS---------- 904
MdAmyF3         VYAAIIDEKVAIKIGPGHYEP-ASGPQNWNKSLEGRDYKVWEAS---------- 901
CsAmyF3         VYAAIIDEKVAMKLGPGHYEP-PSGSQNWCFVTEGRDYKVWEAA---------- 900
MiAmy           VYAAIIDKKVAMKIGPGHYEP-PNGPQKWSFVMEGRDYKIWEAS---------- 594
RcAmyF1         VYAAIIEEKVAMKIGPGHYEP-PSG-KNWSMAIEGKDYKVWEAS---------- 900
DzAmyF3         VYAAIIDEKVAMKIGPGYYEP-PGGPQRWSSVLEGKDYKVWEAC---------- 892
AcAmyF3         VYAAIIDKKVAMKIGPGFYEP-ASGPQRWSLAVEGNDYKVWEAS---------- 895
AcAmyF1         VYAAIIDKKVAMKIGPGFYEP-ASGPQRWSLAVEGNDYKVWEAS---------- 895
GsAmyF1         VYAAIVDDKVAMKIGPGHFEP-PSGSQRWSSALEGRDYKIWEAS---------- 922
AtAmyF3         VYAAIIDEKVAMKIGPGHYEP-PNGSQNWSVAVEGRDYKVWETS---------- 887
MdAmyF1         LYSAMIGEKVCMKIGDGSWCP-AGR--EWTLATCGHRYAVWNK---------- 413
StAmyF1         LYAATIDEKVSVKIGDGSWSP-AGK--EWTLATSGHRYAVWQK---------- 407
OsAmyF1         LYLAEIDGKVITKIGPRYDVE-HLIPEGFQVVAHGDGYAIWEKI---------- 434
HdAmyF1         AYVAEIDGKVVVKIGSRYDVG-AVIPAGFVTSAHGNDYAVWEKNGAAATLQRS- 438
MuAmyF1         LYMAMIDGKILTKLGSRYDVG-NLVPSNFHVVASGNDYCVWEKR---------- 416
TaAmyF1         LYVAKIGGKVITKIGSRYNIGDNVIPSGFKIAAKGNNYCVWEKSGL-------- 413
ZaAmyF1         LYVAKIDDKVIVKIGSRYDVG-NLIPSDFHAVAHGNNYCVWEKHGLRVPAGRHH 439
                 * * :  *:  *:*             :     *  * :*:
```

Fig. 2.A comparison of the amino acid sequences of various plant α-amylases. Amino acid residues are shown as single letters as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Enzyme sources are shown in Table 2. Amino acids 447-460 of plastid α-amylases were removed. The four well-accepted regions I, II, III and IV are surrounded by rectangles. The conserved binding site and active site residues are numbered H291, D372, E397 and D481, which are present in the 4 well-accepted conserved regions I, II, III and IV, respectively. The conserved residues that are thought to be a part of the Ca^{2+} binding site are labeled with "C." The residues involved in hydrogen bonding to the α-amylase inhibitor acarbose are conserved and labeled "Z." A predicted sugar tong binding site in domain C is labeled "S." The names and identifying details for α-amylases are shown in Table 2

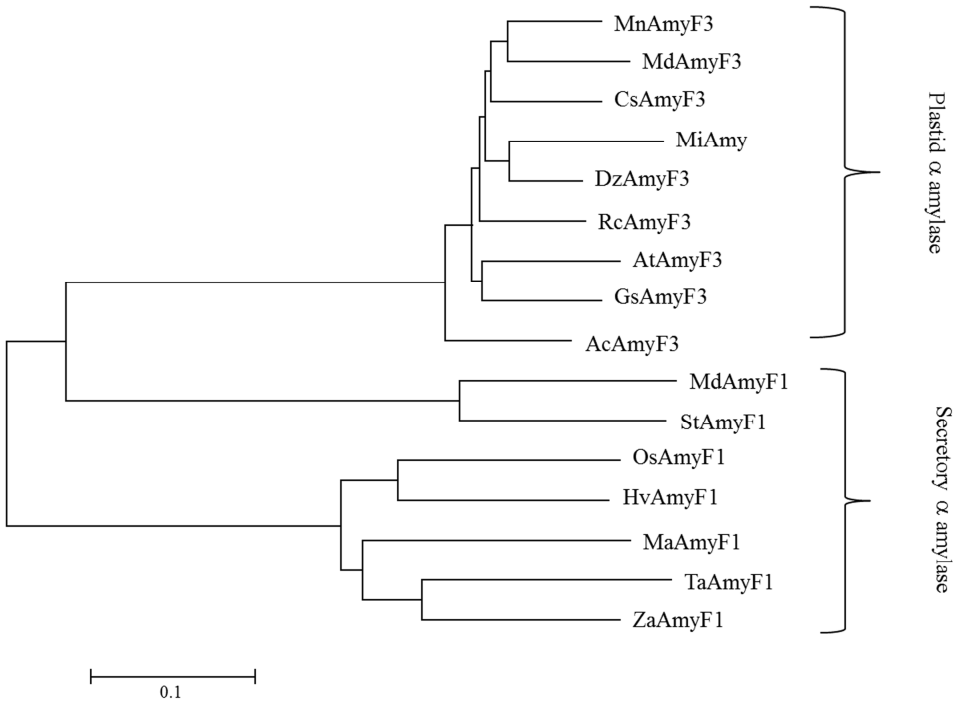

Fig. 3. The phylogenetic tree of MiAmy grouped with other α-amylases from plants

Fig. 4. Sequence alignment of MiAmy and HVAmyF1 were conducted using ClustalW alignment. Secondary structural prediction was performed using InterProScan after 201 residues of MiAmy were removed. The β-strands and α-helices in the catalytic domain of domain A are labelled Aβ1-8 and Aα1-8, B indicates the B domain and C denotes the C-terminal domain. Identical residues are boxed. The active site residues are shown in gray boxes

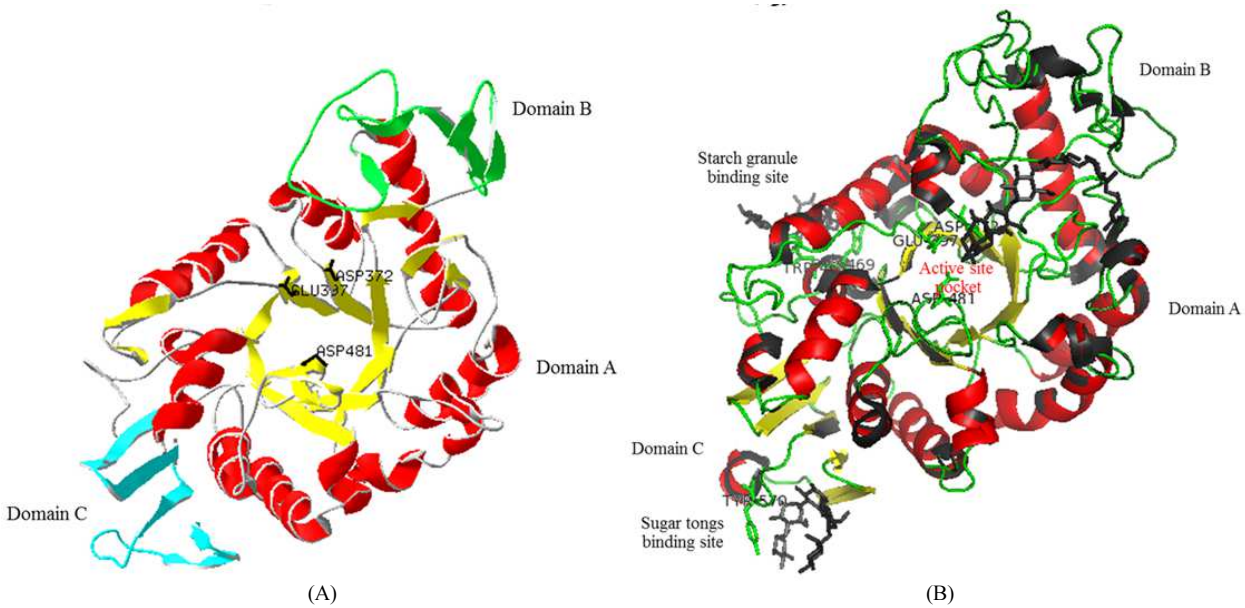

Fig. 5. Predicted 3D structure of MiAmy. A: The predicted 3D structure of MiAmy revealed domain A (red and yellow), B (green) and C (blue) containing D372, E397 and D481 as active site residues using HvAmyF1 (1RP8) as a template. B: A merged structure of α-amylase from Ok-rong mango (color) and template (gray); the structure displayed shows the sugar tong binding site and starch granule binding site as gray sticks

α Amylase Secondary Structure Prediction

InterProScan-an integration platform for the signature-recognition methods in InterPro Bioinformatics was used to determine the positions of helices and extended-chains along the polypeptide chains of plants α amylase. The comparison of secondary structure between MiAmy and the well study HvAmyF1 confirmed that 594 aa contained with active Domain A, substrate binding Domain B and C-terminus Domain C (Fig. 4).

α Amylase Three Dimensional Structure Prediction

For structural grouping, the predicted 3D structure of α-amylase from Ok-rong mango was generated using the SPDBV Swiss model program and Pymol using 1RP8.pdb (crystal structure of barley alpha-amylase isoform 1) as a template (Fig. 5). The partial amino acid sequence of Ok-rong mango α-amylase displayed 42% sequence identity with the X-ray structure of barley α-amylase and Domain A was conserved.

Discussion

In this study, 594-amino acid MiAmy with an active domain, was confirmed to be a plastid α amylase and classified into the family 13. The alignment revealed the difference between secretory α amylases and plastid α amylases. In the fourth region, the protein can be classified into two groups, FVDNHD and FIENHD for secretory and plastid α amylases, respectively. MiAmy region four allowed the protein to be classified into the group of plastid α amylase. However, the difference between VD and IE in this region does not affect the mechanistic properties. In this study, nearly all critical domains and region of MiAmy are found, which are as follows: (1) the two consecutive tryptophan residues for starch-granule binding, which correspond to W469 and W470 (Tangphatsornruang *et al*., 2005), (2) the conserved residues thought to be the Ca^{2+} binding site, (3) the residues involved in hydrogen bonding with the α-amylase inhibitor acarbose, (4) Tyr 570 in domain C, corresponding to Tyr807 of DzAmyF3, which was suggested to be a sugar tongs surface binding site (Posoongnoen *et al*., 2015). Thus, the MiAmy α-amylase gene is predicted to be valuable for further studies, including its modification.

The secondary structure of the catalytic domain of all members, including MiAmy showed the same basic $(\beta/\alpha)_8$ barrel, eight β stands surrounded by eight α helices. Residues 202-532 of MiAmy are homologous with catalytic domains of many members of α amylase family 13 including residues 26-354 of MdAmyF1, 20-348 of StAmyF1, 32-372 of OsAmyF1, 26-376 of HvAmyF1, 16-354 of MaAmyF1, 26-348 of TaAmyF1, 29-367 of ZmAmyF1, 496-825 of AcAmyF3, 513-842 of MnAmyF3, 510-839 of MdAmyF3, 496-825 of CsAmyF3, 496-825 of At3AmyF, 531-860 of GsAmyF3, 510-839 of RcAmyF3 and 501-830 of DzAmyF3 (Fig. 2). This result correspond with the first 380 residues of secretory Taka-amylase A which constitutes the active domain necessary for α-amylase activity (Matsuura *et al*., 1984). For chloroplastid α-amylase, the active domain was characterized as the middle to the C-terminal end. Previous studies reported that an active domain, comprising amino acids 380-440, is necessary for activity of α-amylase (MacGregor, 1988). At least 101 amino acid residues of the C-terminus could be removed without the losing enzyme activity (Yamazaki *et al*., 1983).

Conclusion

An amino acid sequence of α-amylase from Ok-rong mango (*Mangifera indica*), MiAmy, was determined and classified as a plastid amylase. Bioinformatics revealed that only the amino acid residues forming the active domain are important for function and activity. In support of a previous report, 496 aa at the N-terminus of AtAmy3 was removed. The construction of recombinant shortened (390-aa) AtAmy3 with an active domain showed equal activity when compared with the full-length native 887-aa AtAmy3 (Yu *et al*., 2005), which supports that the identified sequence for MiAmy, containing the active domain, is sufficient for cloning and expression.

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Author's Contributions

Raksmont Ubonbal: Conducted all experiments, coordinated the data analysis and drafted the manuscript.

Saijai Posoongnoen: Supervised for sequence determination and manuscript production.

Sompong Klaynongsruang: Concieved the main project idea and guide for manuscript production.

Jureerut Daduang: Guided for bioinformatics analysis.

Sakda Daduang: Designed the experiments, coordinated the study, integrated all the data, verified the result and rewrote the manuscript.

Ethics

The authors declare that there is no conflict of interest.

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