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

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Corresponding Author: Sakda Daduang Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand Email: sakdad@kku.ac.th **Abstract:** The 594-amino acid residue sequence of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -amylase family 13 because it has an active Domain A, similar to a ( $\beta/\alpha$ )<sub>8</sub>-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

 $\alpha$  Amylases are classified into amylase family-13 (EC 3.2.1.1) or GH-13 which hydrolyzes  $\alpha$ -1, 4-D-glucosidic bonds. These proteins are found in diverse organisms and are still being characterized.  $\alpha$  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  $\alpha$  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. Okrong) 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  $\alpha$ -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



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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<sup>TM</sup>). For  $\alpha$ amylase gene amplification, primers (Table 1) were designed from the  $\alpha$  amylase sequences from other plants, Arabidopsis thaliana; AY065233, Malus domestica; AAF63939 and Citrus sinensis; XP\_006483229 (Genbank). Next, specific primers were designed from our  $\alpha$  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**<sup>®</sup> 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 (MEGA6: 60 http://megasoftware.net/) (Tamura et al., 2013). Threedimensional structural predictions were launched using the SPDBV Swiss model program (http://spdbv.vitalit.ch/) (Guex and Peitsch, 1997) and the catalytic region domain was confirmed using the InterProScan Swiss model program (http://swissmodel.expasy.org/workspace/) (Zdobnov and Apweiler, 2001).

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

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

Table 2. a 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  $\alpha$  amylase sequence. The sequence was found to be 1782 base pairs of nucleotides and share 85, 83 and 79% identity with  $\alpha$ 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  $\alpha$  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  $\alpha$ -amylase. The large Nterminal domain of plastid  $\alpha$ -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  $\alpha$ -1, 4 glucosidic bond for  $\alpha$  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 $\alpha$ 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  $\alpha$  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  $\alpha$  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  $\alpha$  amylase from plastids.

	FM1RM1	511 bp
	FM2RM3 435 bp	RM2AUAP 311
	885 bp	
FM3RM4 500 bp		
FM3RM3 1300 bp		

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

	c c
MnAmyF3	QGFNWES-HKVGRWYMELKDKARELSSLGFTVIWLPPPTESVSEQGYMPTDLYNLN-SRY 574
MdAmyF2	OGENWES-SESGRWYEELESKAAELSSLOFTVINEPOPTDSUSDOTWEEDLYNN, GOV 531
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miAmy	WAT REED - REJUKWINELKERAADLISIOFTVIWLPPPTESVSPEGYMPKDLYNLN~SEY 263
RcAmyF1	QGFNWES-NKSGRWHMELKEKAAEISSLGFTVIWLPPPTESVSPEGYMPKDLYNLN-SRY 571
DZAMYF3	QGFNWES-HKSGRWYMELKEKALEISSLGFTVIWLPPPTESVSAEGYMPTDLYNLN-SRY 562
AcAmyF3	QGFNWES-HESGRWYMQLLERAAEISSIGFTVVWLPPPTESVSPEGYMPGDLYNLN-SRY 565
AcAmyF1	QGFNWES-HKSGRWYMQLLERAAEISSIGFTVVWLFFFTESVSFEGYMFGDLYNLN-SRY 565
GsAmyF1	QGFNWES-HRSGRWYMELKEKAAELASFGFTVIWLPPPTESVSPEGYMPKDLYNLN-SRY 592
AtAmyF3	OGFNWES-NKSGRWYLELOEKADELASLGFTVLWLPPFTESVSPEGYMPKDLYNLN-SRY 557
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OsAmyF1	QGFNWESWKENGGWYNFLMGKVDDIAAAGITHVWLPPPSHSVGEQGYMPGRLYDLDASKY 95
HdAmyF1	QGFNWESWEQSGGWYNNMMGKVDDIAAAGVTHVWLPPPSHSVSNEGYMPGRLYDIDASEY 89
MuAmyF1	QGFNWESWRQQGGWYNFLKDKVSDIANAGVTHVWLPPPSHSVGVQGYMPGRLYDLGASKY 79
TaAmyF1	OGFNWESWKTOGGWYKFMOGKVEEIASTGATHVWLFPPSOSVSPEGYLPGOLYNLN-SKY 88
2aAmyF1	OGENWESWKKOGGWYNYLLGRVDDIAATGATHYWLPPPSHSVAPOGYMPGRLYDLDASKY 92
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MnAmyF3	GIMEELKEIVMIPHEVOMKVLGDVVLNH&CAQYQNGVWNVFGGRLNWDDRAIV 629
MdAmyF3	GNMDELKETVKTFHDAGLKVLGDAVLNHACAEYQNQNGVWNIFGGRLNWDEFAVV 626
CsAmyF3	GNIDELKDVVNKFHDVOMKILGDVVLNHLCAHYQNQNGVWNIFGGRLNWDDRAVV 625
MiAmy	GTIDELKDVVNKFHEVGIKVL4DAVLNH#CAHFQNQNGVWNIFGGHLNWDDPAVV 318
RcAmyF1	GSIDELKDLVKSLHRVGLKVLØDAVLNHÅCAHFONONGVWNIFGGRLNWDDRAIV 626
DzAmyF3	GTIDELKELVKNLHDAGLKVLODVVLNHRCAHFONONGVWNIFGGRLNWDDRAVV 617
AcAmyE3	GNVEELKLIVKRFHEVGIRVLGDVVLNHKCAOYKNONGIWNIFGGRLNWDDRAVV 620
AcAmyF1	GNVEELKLIVKBFHEVGIRVLODVVLNH&CAOYKNONGIWNIFGGBLNWDDRAVV 620
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OsAmyF1	GNEAQLKSLIEAFHGKGVQVIADIVINHATAEHKDGRGIYCLFEGGTPDSRLDWGPHMIC 155
HdAmyF1	GNAAELKSLIGALHGKGVQAIADIVINH&CADYKDSRGIYCIFEGGTSDGRLDWGPHMIC 149
MuAmyF1	GNODELKALIGAFHDKGVKCVADIVINHKCADKODGRGIWCIFEGGTDDARLDWGPHMIC 139
TaAmyF1	OSGADLKSLIGAFRGKNISCVADIVINHKCADKKDGRGVYCIFEGGTSDNBLDWGPDEIC 148
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CSAMYE3	ADDP-HFQGRGNKSSGDNFHAAPNIDHSQDFVRKDIKEWLCWLRNEIGYDSWRLDFVRGF 684
MiAmy	CDDF-HFQGRGNKSSGDNFHAAFNIDHSQDFVRKDIKEWLCWLRREIGYDSWRLDFVRG# 377
RcAmyF1	ADDP-HFQGRGSKSSGDNFHAAPNIDHSQDFVRQDLKEWLCWLRDEIGYNGWRLDFVRGF 685
DZAmyF3	GDDP-HFOGRGNKSSGDNFHAAFNIDHSOEFVRKDLKEWLVWLRDEIGYDDWRLDFVRGF 676
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ACAMYE3	ADDP-HFQGRONKSSGDNFHAAPNIDHSQDFVRKDIKEWLCWMREEVGIDGWRLDFVRGF 6/1
MdAmyF1	SCTGGLGNPSTGDNFHGVFNIDHSQLFVRKDITGWLQWLRNNVGFQDFRFDFARGY 198
StAmyF1	SCTGGRGNKSTGDNFNGVPNIDHTQSFVRKDLIDWMRWLRSSVGFQDFRFDFAKG* 192
OsAmyF1	RDDP-YGDGTGNPDTGADFAAAPDIDHLNKRVQRELIGWLDWLKMDIGFDAWRLDFAKG‡ 214
HdAmyF1	RDDTKYSDGTANLDTGADFAAAPDIDHLNDRVQRELKEWLLWLKSDLGFDAWRLDFARGY 209
MUAmyF1	RDDTOYSDGTGNLDTGEGFAAAPDIDHLNTOVOHELTDWLNWLKTDIGFDGWRLDFAKG 199
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AcAmyF3	WGGYIKDYIDASEPYFA¥GEYWD SLSXTYGEMDHNODAHRORIIEWINATSGTAGA 735
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AtAmyF3	WGGYVKDYMDASKPYFAYGEYWD SLSYTYGEMDYNODAHRORIVDWINATSGAAGA 727
MdAmyF1	SARYVKEY IEGAKPIFSTGEYND SCNYNGHGLDYTODSHROPTUNNINGTGOL STA 254
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OsAmyF1	SADMAKIYIDATEPSFA¥AEIWT\$MANGGDGKPNYDQNAHRQELVNWVDRVGGANSNATA 274
HdAmyF1	SPEMAKVY I DOTSPSIAVAEVNDNMATGODGK PNYDODAHRONT UNSUDKUGGAA SAGNY 260
Markenster	
MUAMYF1	SSSIARIIVEQIQPNEVYAEIWSŞLAIRNDGKPTYDQNGNRQGLVNWVQQVGGFVTA 256
TaAmyF1	SAAMAKIYVDNSKPAFVÝGELYDRDROLLANWVRGVGGPATA 250
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MnAmyF2	FDVTTKGTLHSALERCEYWRLSDEKOKPROVOWWRSEAVTFTENHEFOSTOOPWRSEOO. 004
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mciamyr3	FUVIINGLLHAALERGEIWELSDEKGRPPGVLGWWFSEAVTFIENHDIGSTQGHWRFPNK 801
CSAmyF3	FDVTTKGILHSALDRCEYWRLSDEKGKPPGVVGWWPSRAVTFIENHDTGSTQGHWRFPGG 800
MiAmy	FDVSTKGILHSALERCEYWRLSDOKGKPPGVVGWWPSRAVTFIENHDGSTOGHWRFPGG 494
RCAMVE1	FDUTTKGILHSALDBCEYWBLSDOKGKPPGUUGWBSEAUTETENHEFGSTOGUNG FDUG AGI
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UZAMYE3	EDVOINGLENGELENGEIWRLDUGRGRPPGVVGWWPSKAVIFIENHDIGSTGGHWRFPGG 792
AcAmyF3	FDVTTKGILHSALQRCEYWRLSDQKGKPFGVVGWWPSRAVTFIENHDFGSTQGHWRFPGG 795
AcAmyF1	FDVTTKGILHSALQRCEYWRLSDQKGKPPGVVGWWPSRAVTFIENHDTGSTOGHWRFPGG 795
GRAMVE1	FDVTTKGTLHSALSPCEYWPLSDOKCKPPCUT CHMPSPAUTETENHTFOSTOCHUPPPOG 202
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ACAMYE'S	FUVITAGILHTALQACEIWRLSDPKGKPPGVVGWWPSRAVTFIENHDFGSTQGHWRFPEG 787
MdAmyF1	fdfiikgilQEAvkg-QlwrlrDPQGkPPGvvGwwPsrSvTflDnHffGSTQAHwPfPTN 313
StAmyF1	FDFTTKAVLQEAVKG-EFWRLRDSKGKPPGVLGLWPSRAVTFIDNHDIGSTOAHWPFPSR 307
OsAmyF1	FDFTTKGILNVAVEG-ELWELEGEDGKAPGMTGWEAKATTEUDNHEFGSTGHI WEFEED 233
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HCIAMYF1	fuftingilnaaveg-elwelidpogkapgvmgwwpakaatpvdnhmggstgamwpPpsD 328
MuAmyF1	FDFTTKGILQAAVEG-ELWRMRDPQGKAPGMMGNWPEKAVTFVDNHDTGSTQKLWPFPSD 315
TaAmyF1	FDFPTKGVLOEAVOG-DLGRMRGSDGKAPGMIGWMPEKTVTFIDNHDGSTGRIWPFPSD 309
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MnAmyF3	KEIQGYAYILTHPGTPSVFYDHIFSHYGSEIGSLISLRNRNKIHCRSRVQITKAERD	861
MdAmyF3	KEMQGYAYILTHPGTPTVFYDHIFSHYQSEIAALISLRNRNKLNCRSRVKITKAERD	858
CSAmyF3	REMOGYAYILTHPGTPSVFYDHIFSHYRQEIEALLSVRKRNKIHCRSRVEIVKAERD	857
MiAmy	KELQGYAYILTHPGTPAVFYDHIFSNYRSEIEALISLRKRNKLHCRSIVQIAKAERD	551
RcAmyF1	KEMQGYAYILTHPGTPTVFYDHIFSHYRSEIASLISLRKRNEIHCRSSVKITKAERD	858
DzAmyF3	KELQGYAYILTHPGTPAVFYDHISSHYRSEIAALISLRNRNKIHCRSTVKIVKAERD	849
AcAmyF3	KEMQGYAYILTHPGTPAVFYDHAFHHMRSEISALVSLRNRNKIHCRSTIQITKAERD	852
AcAmyF1	KEMQGYAYILTHPGTPAVFYDHAFHRMRSEISALVSLRNRNKIHCRSTIQITKAERD	852
GsAmyF1	KEMQGYAYTLTHFGTPSVFYDHIFSHYKTEIATLISIRKRNKIHCRSTLKICKAERD	879
AtAmyF3	KEMQGYAYILTHPGTPAVFFDHIFSDYHSEIAALLSLRNRQKLHCRSEVNIDKSERD	844
MdAmyF1	HIMEGYTYILTHPGIPTVFYDHFYDWGDSIHDQIVKLIDIRKRQDIHSRSSITILEAQPN	373
StAmyF1	HVMEGYAYILTHPGIPSVFFDHFYEWDNSMHDQIVKLIAIRRNQGIHSRSSIRILEAQPN	367
OsAmyF1	KVMQGYAYILTHPGNPCIFYDHFFDWGLKEEIERLVSIRNRQGIHPASELRIMEADSD	391
HdAmyF1	KVMQGYAYILTHPGIPCIFYDHFFNWGFKDQIAALVAIRKRNGITATSALKILMHEGD	386
MuAmyF1	KVMQGYAYILTHPGVPSIFYDHMFDWGLKEKITRLAKTRTRNRIHSGSSLNILASDAD	373
TaAmyFl	KVMQGYAYILTHPGIPCIFYDHVFDWKLKQEITALATVRSRNGIHPGSTLDILKAEGD	367
ZaAmyF1	KVMQGYAYILTHPGTPCIFYDHVFDWNLKQEISALSAVRSRNGIHPGSELNILAADGD	386
	s	
MnAmyF3	VYAAIIDEKVAVKIGPGHYEP-PSGPQRWSRAVEGRDYKVWEAS 904	
MdAmyF3	VYAAIIDEKVAIKIGPGHYEP-ASGPONWNKSLEGRDYKVWEAS 901	
CsAmyF3	VYAAIIDEKVAMKLGPGHYEP-PSGSONWCFVTEGRDYKVWEAA 900	
MiAmy	VYAAIIDKKVAMKIGPGHYEP-PNGPOKWSFVMEGRDYKIWEAS 594	
RcAmyF1	VYAAIIEEKVAMKIGPGHYEP-PSG-KNWSMAIEGKDYKVWEAS 900	
DzAmyF3	VYAAIIDEKVAMKIGPGYYEP-PGGPQRWSSVLEGKDYKVWEAC 892	
AcAmyF3	VYAAIIDKKVAMKIGPGFYEP-ASGPORWSLAVEGNDYKVWEAS 895	
AcAmyF1	VYAAIIDKKVAMKIGPGFYEP-ASGPORWSLAVEGNDYKVWEAS 895	
GsAmyF1	VYAAIVDDKVAMKIGPGHFEP-PSGSQRWSSALEGRDYKIWEAS 922	
AtAmyF3	VYAAIIDEKVAMKIGPGHYEP-PNGSQNWSVAVEGRDYKVWETS 887	
MdAmyF1	LYSAMIGEKVCMKIGDGSWCP-AGREWTLATCGHRYAVWNK 413	
StAmyF1	LYAATIDEKVSVKIGDGSWSP-AGKEWTLATSGHRYAVWCK 407	
OsAmyF1	LYLAEIDGKVITKIGPRYDVE-HLIPEGFQVVAHGDGYAIWEKI 434	
HdAmyF1	AYVAEIDGKVVVKIGSRYDVG-AVIPAGFVTSAHGNDYAVWEKNGAAATLORS- 438	
MuAmyF1	LYMAMIDGKILTKLGSRYDVG-NLVPSNFHVVASGNDYCVWEKR 416	
TaAmyF1	LYVAKIGGKVITKIGSRYNIGDNVIPSGFKIAAKGNNYCVWEKSGL 413	
ZaAmyF1	LYVAKIDDKVIVKIGSRYDVG-NLIPSDFHAVAHGNNYCVWEKHGLRVPAGRHH 439	
	* * : *: *:* : * * :*:	

Fig. 2.A comparison of the amino acid sequences of various plant  $\alpha$ -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  $\alpha$ -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<sup>2+</sup> binding site are labeled with "C." The residues involved in hydrogen bonding to the  $\alpha$ -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  $\alpha$ -amylases are shown in Table 2



Fig. 3. The phylogenetic tree of MiAmy grouped with other  $\alpha$ -amylases from plants

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	Αβ1	Aal www.ww	Αβ2	
MiAmy HvAmyF1	TAEPEISEPSPKVSSGTGTGFEILCQGFNWESHK-SGRWY	MELKEKAADL NMMMGKVDDI	SSLGFTVIWL	239 64
	Αα2 www.www.ww	Αβ3		
MiAmy	PPPTESVSPEGYMPKDLYNLN-SRYGTIDELKDVVNKFHE	VGIKVLGDAV	LNHRCAHFQN	298
HvAmyF1	PPPSHSVSNEGYMPGRLYDIDASKYGNAAELKSLIGALHG	KGVQAIADIV	INHRCADYKD	124
	В			
MiAmv	QNGVWNIFGGHLNWDDRAMVCDDPHFQ-GRGNKSS	GDNFHAAPNI	DHSQDFVRKD	352
HvAmyF1	SRGIYCIFEGGTSDGRLDWGPHMICRDDTKYSDGTANLDT	GADFAAAPDI	DHENDRVQRE	184
	$A\alpha^3$ $A\beta^4$ $A\alpha^4$ $A\alpha^4$	Αβ5		
MiAmy	IKEWICWLRREIGYDGWRLDFVRGEWGGYVKDYLDASEPY	FSVGEYWDLS	IKYTYGEMDH	412
HvAmyF1	LKEWILWLKSDLGFDAWRLDFARGYSPEMAKVYIDGTSPS	LAVAEVWDNM	ATGGDGKPNY	244
	Αα5 Αβ6 Αα6	Aa6 WWW		
MiAmy	NQDAHRQRIIDWINATNGTAGAFDVSTKGILHSALER	CEYWRLSDQK	GKPPGVVGWW	469
HvAmyF1	DQDAHRQNLVNWVDKVGGAASAGMVFDFTTKGILNAAVEG	ELWRLIDPO	GKAPGVMGWW	303
	Αβ7 Αα7	Αβ8	Aa8	
MiAmy	PSRAVTFIENHDTGSTQGHWRFPGGKELQGYAYILTHPGT	PAVFYDHIFS	N-YRSEIEAL	528
HvAmyF1	PAKAATFVDNHDTGSTQAMWPFPSDKVMQGYAYILTHPGI	PCIFYDHFFN	WGFKDQIAAL	363
	Aa8 C			
MiAmy HvAmyF1	ISLRKRNKLHCRSIVQIAKAERDYYAAIIDKKVAMKIGPG	HYEPPNGPQK	WSFVMEGRDY	588
	VAIRKRNGITATSALKILMHEGDAYVAEIDGKVVVKIGSR	YDVGAVIPAG	FVTSAHGNDY	423
	$\longrightarrow$			
MiAmy	KIWEAS 594			
HvAmyF1	AVWERNGAAATLQRS 438			

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

## a 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  $\alpha$  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).

#### $\alpha$ Amylase Three Dimensional Structure Prediction

For structural grouping, the predicted 3D structure of  $\alpha$ -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  $\alpha$ -amylase displayed 42% sequence identity with the X-ray structure of barley  $\alpha$ -amylase and Domain A was conserved.

## Discussion

In this study, 594-amino acid MiAmy with an active domain, was confirmed to be a plastid  $\alpha$  amylase and classified into the family 13. The alignment revealed the difference between secretory  $\alpha$  amylases and plastid  $\alpha$  amylases. In the fourth region, the protein can be classified into two groups, FVDNHD and FIENHD for secretory and plastid  $\alpha$  amylases, respectively. MiAmy region four allowed the protein to be classified into the group of plastid  $\alpha$  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  $\alpha$ 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  $\alpha$ 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  $\beta$  stands surrounded by eight  $\alpha$  helices. Residues 202-532 of MiAmy are homologous with catalytic domains of many members of  $\alpha$  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  $\alpha$ -amylase activity (Matsuura *et al.*, 1984). For chloroplastid  $\alpha$ -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  $\alpha$ -amylase from Okrong 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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