ISOLATION OF cDNA FRAGMENT ENCODING ISOAMYLASE GENE FROM METROXYLON SAGU BY RT-PCR METHOD

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ISOLATION OF cDNA FRAGMENT ENCODING ISOAMYLASE GENE FROM
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Isolation of cDNA fragment encoding Isoamylase gene from *Metroxylon sagu* by RT-PCR method

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**ABSTRACT**

Isoamylase is one of starch debranching enzymes (DBEs) that involves in starch biosynthesis. The study of isoamylase gene from *Metroxylon sagu* (sago palm), which considered very productive in starch production, could provide a better insight in understanding the gene properties and its function. Isoamylase gene have been characterized in maize, rice, potato, barley and wheat in previous study but it is yet to be explain in sago palm. For identifying isoamylase gene in sago palm, total RNA was extracted and RT-PCR technique was performed. cDNA was synthesized and simultaneously amplified by RT-PCR method, which offers rapid, most sensitive and versatile way in analyzing gene expression. Isoamylase degenerated primers were design for priming specific gene in PCR reaction. Nevertheless, in this study, the isolation of fragment encoding isoamylase gene in sago palm was not achieved as the full length of expected isoamylase gene fragment was not obtained.

Key words: *Metroxylon sagu*, isoamylase gene, starch debranching enzymes, RT-PCR, degenerated primers

**ABSTRAK**

Isoamylase adalah salah satu daripada starch debranching enzyme (DBEs) yang terlibat dalam proses penghasilan kanji. Kajian tentang gen isoamylase daripada *Metroxylon sagu* (sagu), yang dianggap sangat produktif dalam penghasilan kanji boleh membantu dalam memahami ciri-ciri dan fungsi gen tersebut. Ciri-ciri gen isoamylase pada jagung, padi, kentang, barli dan gandum telah dikaji dalam kajian sebelum ini tetapi ianya belum dijalaskan dalam sagu. Dalam mengenalpasti gen isoamylase pada sagu, RNA diekstrak dan teknik RT-PCR dijalankan. cDNA disintesis dan diperbanyakkan secara serentak melalui teknik RT-PCR yang menawarkan cara yang cepat, paling sensitif dan versatile untuk mengkaji pengekspresan gen. Degenerated primer isoamylase dihasilkan untuk mengenalpasti gen sasaran dalam tindak balas PCR. Walau bagaimanapun, dalam projek ini, pengasingan fragmen yang mengkodkan gen isoamylase dalam sagu tidak dicapai disebabkan fragmen gen isoamylase tidak diperolehi.

Kata kunci: *Metroxylon sagu*, gen isoamylase, starch debranching enzymes, RT-PCR, degenerated primers
CHAPTER 1

INTRODUCTION

1.1 Introduction

Metroxylon sagu (M. sagu), commonly known, as sago palm is considerably very productive in starch compared to other Metroxylon species namely M. amicarum, M. warburgii and M. salomonense (McClacthey et al., 2004). Hence, sago palm has been use for its starch as staple food in human diet for years especially in Southeast Asia where it found endemically.

In Malaysia, sago palm grown commercially and the largest sago growing areas found in the state of Sarawak. This contributes to intensive use of sago starch as it has been commercialize mainly in East Malaysia, Sarawak and it became of importance for international exports (Flach, 1997) and made Sarawak among the biggest exporter of sago and its products mainly to Peninsular Malaysia, Japan, Taiwan, Singapore and several other countries according to Malaysian Agricultural Economics Association. Sago palm was not only used for the production of glucose but also important as a generic fermentation medium (Abd-Aziz, 2002). As Sarawak has diverse amount of sago palm, therefore the study of genes that involves in its system would be treasure for better understanding and effectively utilize of this local crops and as well as to retain its diversity.

Starch biosynthesis involved various types of enzymes. Martin and Smith (1995), have described the biochemistry of starch biosynthesis, which involved the crucial enzymes that
committed to starch production. These include ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS) and starch-branching enzymes (SBEs) as well as starch debranching enzyme (DBEs).

The main concern in this study was on DBEs. There were two types of DBEs that occur in higher plants namely, isoamylase-type and pullulanase-type. Isoamylase is a type of DBEs that undergone vast sums of research studies as an effort to explain its exact function mainly during determination of amylopectin structure that involves the crystallization of glucan granule. As sago palm is a very high productive in starch (McClacthey et al., 2004), it should be convenient to study such enzyme as preference to reveal its properties accordingly by using the advance technology of bioinformatics in conducting research. Since isoamylase gene have been sequence from other starch producing plant in previous studies, this information could be useful in sequence analysis purpose.

The study of gene requires the extraction of DNA or RNA as crucial starting materials prior any other manipulation to subsequently done. Thus, in this study, to obtain more stable molecule for further analysis, complementary DNA (cDNA) was reverse transcribe from mRNA and use as template (Brown, 2001). Nevertheless, cDNA allows one to find for specific gene as it comprises of only coding region (Bourgaize et al., 1999) and can generates directly from total RNA by using specific design primers.

Moreover, it would be a cornerstone for prediction of genes structure and function by first identify the gene and isolate the fragments that encoded for isoamylase and get the correct
gene sequence. For that reason, degenerated primers were design to obtain the gene target from the cDNA template of sago palm using Reverse Transcription – Polymerase Chain Reaction (RT-PCR) method.

RT-PCR combines cDNA synthesis from RNA template with PCR to provide rapid, versatile and sensitive method to analyze gene expression and making it leading RNA analysis technique use in current research (Montarras et al., 1994). The advantage of using this technique is it is able to detect and quantify the expression of messages from small amount of RNA sample. There are two formats of RT-PCR, which can be carried out either using two-steps or one-step format. Choosing the right format could be an advantage as in one-step format, the carry-over contamination can be minimized and yet, it may very useful to carry out large number of samples (Lee et al., 2003).

Figure 1. RT-PCR overview. cDNA synthesis can be perform by using either gene-specific primer, oligo (dT) primer or random hexamer primers and latter the fragments will be amplified in PCR reaction. (Adapted from Lee et al., 2003).
1.2 Objectives

This study was conducted mainly to gain knowledge regarding isoamylase gene in sago palm. There are four main objectives that include (1) to generate isoamylase gene specific primers (2) to isolate total RNA for RT-PCR (3) to optimize RT-PCR condition for identifying and amplification of cDNA fragments encoding isoamylase gene (4) to obtain the correct DNA sequence of isoamylase gene that can be use for sequence analysis.

1.3 Research question and study rationale

In previous and current scientific research, DBEs have undergone vast sum of research as to reveal the precise way of the involvement of DBEs in starch biosynthesis specifically during amylopectin synthesis. Due to these progressive study, many starch producing plant been studied and therefore contributes to characterization of genes encode for enzymes that essential in starch biosynthesis. Nevertheless, there is less known of such gene encode for isoamylase been characterized in sago palm. Therefore, this study would give an insight to unravel much information about isoamylase gene in particular and sago palm generally.
CHAPTER 2

LITERATURE REVIEW

2.1 *Metroxylon sagu* (Sago palm)

*Metroxylon sagu* (*M. sagu*) belongs to the family of *Palmae*. The sago palm (*M. sagu*) is widely planted in Sarawak specifically in swampland areas near the rice fields and dwellings (Pearce, 1991). The advantage of this palm is its ability to grow and survive in adverse environments including acid soil, peat soil, submerged soil, and saline soil. Moreover, Osaki et al. (2004) proved that the growth condition, that is different types of soil, might affect the starch properties of sago palm. Hence, McClatchey et al. (2004) noted that, *Metroxylon sp.* grow best in soils with impeded drainage, or with seasonal water logging as they can persist on well drained and in soil that periodically inundated with salt water as long as fresh water flow is more prevalent.

For years, sago palm has been the main subsistence crop especially for several local communities in Sarawak namely, Melanau whose noted the local name for sago palm as *balau* (McClacthey et al., 2004). Other common names for *M. sagu* includes *rumbia* (Malay), *mulong* (Iban, Sarawak), *pohon sagu, pohon rumbia* (Indonesia) and *sakhu* (Thailand) (Pearce, 1991; McClacthey et al., 2004).
Sago palm has been proven as an alternative edible starch as in Sarawak itself, sago pearls are popularly being prepared traditionally for daily consumption mixed with rice bran while in West Malaysia, sago pearls often used to prepare palm pudding (Flach, 1997).

Sago palm found endemically in Papua New Guinea and believed to be endemic in Molucca Islands as well. Nevertheless, Flach (1997) stated that Papua New Guinea was considered as the center of diversity of sago palm. Current distribution of sago palm include Malaysia, Indonesia, Philippines, Papua New Guinea and other countries of Southeast Asia where most of this distribution was meant to be a semi-cultivated stands (McClacthey et al., 2004).

This hapaxantic palm, that is, its flower once and dies thereafter, may reaches 15 m (49 feet) in height with bole diameter of 35-60 cm (14-24 inch). The palm is also soboliferous, that is, it produces tillers or suckers that formed from the lowest part of the trunk, forming a cluster in various stage of development (Flach, 1997).

Figure 2. *M. sago* grow in swampy areas and may reaches 49 feet or more in height. (Adapted from McClacthey et al., 2004).
2.2 Starch properties of sago palm

Starch present in plants granule as a mixture of amylose and amylopectin (Whistler and Daniel., 1984) and it is a common storage homoglycan of glucose in plants. Amylose is an unbranched polymer, which existed, in linear chains that connected by α (1→4) glucosyl linkages. In contrast, amylopectin comprised of linear chains which likewise, formed via α (1→4) glucosyl linkages but its structure was branched by another linkage; α (1→6) glucosyl bonds (Horton et al., 2002).

Branching occurs in average of once in every 25 residues and its side chains may contain 15-25 glucose residues and in addition, some of these side chains are branched as well. The branches produce a compact structure, and provide multiple chain ends with only one reducing ends and plenty of nonreducing ends at which enzymatic cleavage of the polymer and enzymatic lengthening can occur (Horton et al., 2002).

As reported by Ito et al. (1979), sago starch is made up of 27% of unbranched amylose and 73% branched polysaccharides of amylopectin. Kawabata et al. (1984) in contrast, reported that they found a slightly less amount of amylose content of sago starch of only 21.7%. Sago palm could produce starch up to about 250 kg per palm. The sago starch was stored in its bole in the central parenchyma, about 10-25% of its fresh weight of 1-2 tonnes (Flach, 1997). The stored sago starch is crucial meant for the production of sago palm flowers as well as its fruit.
2.3 Starch Biosynthesis

Starch is the most commonly stored carbon in plants where it is specifically located in granules. Produced naturally resulting from reduced carbon which is formed during photosynthesis in plant leaves, starch is a polymer of polysaccharides that required the involvement of crucial enzymes in its biosynthesis metabolism (Horton et al., 2002).

Starch is synthesized via committed three enzymes reaction of ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS) and starch-branching enzymes (SBEs) (Martin and Smith, 1995). Starch is also synthesized transiently in other organs include meristems or root cap cell but its major site of accumulation is in storage organs, which commonly, seeds, fruits, tubers and storage roots.

Synthesis of starch begins when AGPase generates ADP-glucose from glucose-1-phosphate and ATP (liberating pyrophosphate). AGPase are responsible for the synthesis of the substrate for starch biosynthesis in all plant tissues (Smith et al., 1997). ADP-glucose is used by starch synthases as a substrate, which adds the glucose units to the end of growing polymer chain that inevitably, build up a starch molecule by releasing the ADP in the process. SS exists in two distinct forms, which are soluble starch synthase (SSS) and granule bound starch synthase (GBSS). SSS responsible for amylopectin biosynthesis and on the other hand, GBSS is committed for amylase synthesis. Eventually, branches are introduced by SBEs where α-(1→6) glucosyl bonds are inserted into α-(1→4) glucosyl linkages and resulting to the highly
The starch synthesis pathway was presented in Figure 3 below.

Although the pathway of starch biosynthesis appears relatively simple, it is much complicated as it seems when the enzymes that involve come in various different forms, which inevitably differ in their behaviour, and in different parts of plant where they are actively involves (Gray, 2003). The complexity of starch synthesis was created by presence of several other enzymes involves in the pathway. DBEs, the additional enzyme that noted to be involve in starch biosynthesis, hydrolyses α-(1→6) glucosyl bonds and break apart the polymer chain eventually may influence the types of glucan produced (Martin and Smith., 1995). It means that DBEs likewise play an essential role in starch biosynthesis.

![Figure 3. Stages in starch biosynthesis (Adapted from Gray, 2003). ADPase catalyzes the formation of ADP-glucose. SS add glucose units from ADP-glucose to nonreducing end of growing glucan chain and release ADP. SBEs break α-(1→4)-linked glucan chain and form α-(1→6)-linked glucan to form branches and also been shown in stages, was the involvement of DBEs in the pathway (Martin and Smith, 1995).](image-url)
2.4 Starch debranching enzymes (DBEs)

Starch DBEs have been identified in higher plants in two distinct types. There are pullulanase and isoamylase. These two enzymes can be differentiated by their preferences of substrates. Pullulanases hydrolyze most actively on yeast glucan pullulan and they also active on amylopectin and β-limit dextrin but do not hydrolyze glycogen (Nakamura, 1996). It is distinct as isoamylase-type DBEs shows its greatest activity on amylopectin. Likewise, isomylases hydrolyze on both glycogen and β-limit dextrin substrates (James et al., 1995). In this study, the focus is mainly concerns of isoamylase debranching enzyme.

The evidence of DBEs involved in starch synthesis was proved by the early studies of mutant cereals, mutant sugaryl (sul) of maize, rice endosperm and the sta7 mutant of Chlamydomonas reinhardtii indicates strongly that they are important in starch synthesis when those mutant accumulate highly branched and soluble glucan primer (Mouille et al., 1996; Nakamura, 1996).

Nevertheless, the precise and exact roles of both DBEs were remains to be discovered. However, most commonly described for starch DBEs was in the degradation of starch. Much research on cereals, developing pea embryos, mutations affecting these enzymes, potato tubers and maize endosperm shows a consistency with fundamental role for DBEs in starch synthesis. (James et al., 1995; Zhu et al., 1998).
In 1996, Ball and his colleagues have suggested that the synthesis of amylopectin and its organization to form a starch granule involves the ‘trimming’ by DBEs of highly branched, phytoglycogen-like material that synthesized by SS and SBEs at the surface of the starch granule. As the preamylopectin increases in size, DBEs cleave much widely spaced branched to generate such regularly branched glucan structure that competent to crystalline. As mutants are deficient of DBEs, the starch granules are degraded as they formed and exist in an alternative polymer, phytoglycogen resulting from prevented crystallization of glucan.

Moreover, the importance of debranching enzyme activity to amylopectin synthesis was revealed when James et al. (1995) demonstrated the sul locus of maize encodes for a starch DBEs. The similar phenomenon was discovered on the sugary mutant phenotype in rice (Kubo et al., 1999) and the notch2 phenotype in barley (Burton et al., 2002) as both show reduced starch synthesis and the accumulation of phytoglycogen in their endosperm.
2.5 Isoamylase-type DBEs

During starch biosynthesis, the $\alpha-(1\rightarrow6)$ glucosyl linkages that branched amylopectin structures were hydrolyzed by the activity of isoamylase-type of DBEs. Kubo et al. (1999) found that isoamylase plays a predominant role in amylopectin biosynthesis in rice endosperm as pullulanase is also essential or can compensate for the role of isoamylase in the construction of the amylopectin multiple-cluster structure. The super-cluster structure of amylopectin (Gallant et al., 1997) however will be entered the stage where the DBEs will be selectively remove the branched linkages in final determination of amylopectin structure (Ball et al., 1996).

In previous and current scientific researches, Isoamylase gene from several starch producing plants have been identified and characterized. Those include maize, rice, Arabidopsis, barley, potato and wheat (James et al., 1995; Rahman et al., 1998; Zeeman et al., 1998b; Fujita et al., 1999; Borton et al., 2002; Hussain et al., 2003; Rahman et al., 2003). In Arabidopsis, there were three isofonn of isoamylase-type have been identified where as in wheat, expression of cDNA for isofonn of an isoamylase-type DBEs (Iso-1) is indicated and highest in developing endosperm compared to mature grains which was undetectable. This suggests the biosynthetic role for isoamylase in this tissue and in starch biosynthesis generally.
2.6 Complementary DNA (cDNA) synthesis

In higher organisms, specifically plants and animals, their genetic make up are much complicated in terms of their abundant genes and various complexes molecular pathway. Much component is involved in those pathways and it’s originated from the DNA itself. Thus, not all genes in the higher multicellular organisms are expressed at the same time (Brown, 2001). Accordingly, in different cell types both in plants and animals for instance, there are sets of genes, which are relatively, expressed in specialized individual cells types. This regards to the specific gene that active in a specific cell types whereas others are considered to be silent genes (Griffiths et al., 1999; Brown, 2001).

The active genes in those particular cell types will be useful for mRNA purification thus it will inevitably use in cDNA synthesis. The resulting synthesized mature mRNA contains only coding sequences and of introns due to splicing process of transcript primer. This mature mRNA will be used for cDNA synthesis as a template and synthesize by the actions of reverse transcriptase enzyme. The process followed by DNA polymerase activity to synthesis double stranded cDNA (ds cDNA).

The advantageous of utilizing cDNA compared to total genomic DNA to identify specific gene that is, the mRNA that transcribed from particular gene is more abundant than the gene itself in certain tissue or cells (Bourgaize et al., 2000). However, instead of purifying mRNA, the total RNA also can be use as templates for direct first strand cDNA synthesis (Griffiths et al., 1999; Lee et al., 2003).
2.7 RT-PCR

Many techniques have been developed to measure gene expression, those included Northern blots analysis, RNase protection assay, in situ hybridization, dot blots and S1 nuclease assays. Of these techniques, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) offers rapid, most sensitive and versatile way of analyzing gene expression (Montarras et al., 1994). The technique was basically can be used to estimate the expression levels, to determine the presence or absence of a transcript also to clone cDNA products without necessity of constructing and screening a cDNA library.

RT-PCR employed the reverse transcriptase originally from Avian Myoblastosis Virus (AMV) or Moloney murine leukemia virus (M-MLV) being used for synthesizing the first strand of cDNA, a DNA copy of the RNA template, by using either random primers, oligo (dT) primer or sequence-specific primer. Second strand cDNA synthesis and subsequent PCR amplification will be perform with Taq DNA polymerase, which originated from the bacterium Thermus aquaticus (Griffiths et al., 1999). Alternatively, some thermostable DNA polymerases such as Tth DNA polymerase also possess a reverse transcriptase activity, but require manganese instead of magnesium as a cofactor unlike for the Taq DNA polymerase (Myers and Gelfald, 1991).

RT-PCR also permits the simultaneous analysis of a large number of mRNAs from small numbers of cells. This would be important in situation where only small numbers of cells of interest are available that cannot be propagated (Newton and Graham, 1997). The quality and
purify of the stating RNA template is crucial to the success of RT-PCR as either total RNA or poly (A)+ RNA (Griffith et al., 1999) can be used as the starting materials.

2.8 Bioinformatics tools

Bioinformatics tool has tremendously contributes a large impact towards the study of molecular biology. Various bioinformatics software have been developed either exist as freeware, licensed software, shareware or open source software, as well as numerous database available. All of these facilitate in inferring the undiscovered information of biological data of molecular biology.

Major research efforts in the field of bioinformatics include sequence alignment, gene finding, protein structure alignment, protein structure prediction, modeling of evolution etc. ClustalW is one of the standalone multiple alignment program (Higgins et al., 1994). ClustalW program was available at European Bioinformatics Institute online service at http://www.ebi.ac.uk/clustalw/. The basic information they offer includes identification of conserve regions and generates a true phylogenetic tree.

Seqtools is a freeware software that developed by Rasmussen and colleagues (2002) specifically for protein and nucleotides sequences analysis, primer design and protein translation. The software is accessible at http://www.dnatools.org/seqtools.htm. NCBI (National Centre of Biotechnology Information) provide sequences database that can be access at http://www.ncbi.nlm.nih.gov/. NCBI managed the GenBank database that freely available to public.
CHAPTER 3

MATERIALS AND METHODS

3.1 Primer design

3.1.1 Sequences analysis

The amino acid sequences of isoamylase gene were retrieved from NCBI GeneBank online database at http://www.ncbi.nlm.nih.gov. Isoamylase gene sequence of rice (Oryza sativa, Accession no. BAA29041), barley (Hordeum vulgare, Accession no. AAM46866), potato (Solanum tuberosum, Accession no. AY132998) and wheat (Triticum aestivum, Accession no. AAP44580) (Fujita et al., 1999; Burton et al., 2002; Hussain et al., 2003; Rahman et al., 2003) were retrieved. The sequences were initially converted into FASTA format (Pearson and Lipman, 1988; Pearson, 1990) prior to alignment.

ClustalW program (Higgins, 1994), which provides tools to perform multiple sequence alignment, available online at EBI website; http://www.ebi.ac.uk/clustalw/ is used for sequence analysis. The aligned sequences were analyzed to retrieve the conserved regions of isoamylase among these plant species.

Once the conserved regions were retrieved, the amino acid sequences were translated into nucleotides sequences to calculate the melting temperature on each possible sequence for designing primers. Seqtools software which developed by Rasmussen and his colleagues (2002) was used for protein translation.