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Isolation and characterization of protein differentially expressed during oil palm mesocarp development

Isolasi dan karakteristik protein terekpresi secara diferensial selama perkembangan mesokarp pada kelapa sawit

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Ringkasan

Pada kelapa sawit, mesokarp merupakan jaringan yang lebih dikhususkan untuk mensintesis minyak. Akumulasi minyak pada jaringan ini terjadi selama perkembangan buah. Beberapa enzim yang terlibat dalam biosintesis minyak tampaknya disintesis hanya pada periode tertentu dari biosintesis minyak. Sedangkan protein regulator diduga ada pada saat minyak mulai disintesis atau beberapa saat sebelumnya. Sebagai bagian dari usaha mengklon gen kunci untuk biosintesis minyak, penelitian ini bertujuan mengidentifikasi dan mengisolasi protein yang terekspresi secara diferensial sesuai perkembangan buah. Sebagai bahan penelitian digunakan jaringan mesokarp dari berbagai umur buah sawit. Untuk setiap fase perkembangan buah dilakukan analisis kandungan minyak dan protein total. Elektroforesis gel poliakrilamid-SDS (SDS – PAGE) dan elektroforesis dua dimensi (2-D) digunakan untuk mempelajari dan mendeteksi adanya pita protein spesifik yang terekspresi secara diferensial sejalan dengan peningkatan kandungan minyaknya. Hasil penelitian menunjukkan bahwa minyak mulai aktif disintesis pada saat buah berumur 17 minggu setelah antesis. Konsentrasi protein total tidak meningkat sejalan dengan peningkatan kandungan minyaknya. Dari hasil SDS-PAGE terdeteksi dua protein, yaitu protein dengan berat molekul (BM) 31,0 kDa dan 34,3 kDa yang meningkat ekspresinya pada awal dan menjelang periode aktif sintesis minyak. Analisis lebih lanjut dengan elektroforesis 2-D menunjukkan bahwa protein 31,0 kDa terdiri dari dua protein dengan pI 4,64 dan pI 4,95, sedangkan protein 34,3 kDa merupakan protein tunggal dengan pI 4,56. Sikuensing secara parsial kedua protein tersebut menunjukkan adanya dua polipeptida dari protein 31,0 kDa yang mempunyai homologi tinggi dengan subunit biotin karboksilase ht-ACCase, dan empat polipeptida yang mempunyai homologi dengan enoil-ACP reduktase. Sedangkan protein 34,3 kDa mempunyai homologi dengan gliseraldehida 3-fosfat dehidrogenase.

Summary

In oil palm, mesocarp is tissue specialized for oil synthesis. Accumulation of oil in this tissue occurs during fruit development. It is likely that some enzymes involved in oil biosynthesis are synthesized only in a certain period of oil biosynthesis, while regulatory proteins may present at the beginning or right before the period of active oil synthesis. As a part of research work on cloning of gene encoding key enzymes for oil biosynthesis in palm mesocarp, this research was aimed to identify and isolate proteins differentially expressed during fruit development. Mesocarps from different develop-

mental stage of fruit were used for analysis of oil content and protein concentra-tion. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and two dimentional (2-D) electrophoresis were used to study and detect specific protein bands differentially expressed during fruit development. It was shown that oil synthesis was started at 17 weeks after anthesis (WAA). There was no correlation between concentrations of total protein with oil content during mesocarp development. From the SDS-PAGE, two protein of 31.0 kDa and 34.3 kDa were detected that their expression increased at the beginning and just before the period of active oil biosynthesis respectively. Further analysis with 2-D electrophoresis showed that 31.0 kDa-protein consist of two proteins, with pI 4,64 and pI 4,95, while 34.3 kDa protein is a single protein with pI 4,56. Partial amino acid sequencing data of the 31.0 kD protein showed that two polypeptides highly homologous with ht-ACCase biotin carboxylase subunit and four polypeptides homologuus with enoyl-ACP reductase, whereas 34.3 kD protein showed homology with glyceraldehyde-3 phosphate dehydrogenase.

[*Keywords*: Marker protein, oil biosynthesis, oil-palm mesocarp]

Introduction

Gene expression in higher plant takes place through a very precise regulation. A part from the housekeeping genes that encode products required by all cells, each tissue expresses a unique set of structural genes. This set of gene might be expressed in a specific tissue in a certain develop-mental stage or followed an environmental stimulation. This specific gene expression is controlled by *cis*-acting regulatory sequences and *trans*-acting regulatory factors, each of that factor controls gene expression at the transcription and post transcription level (Abdullah, 1994).

In oil palm, mesocarp is tissue specialized for oil synthesis. In this tissue, accumulation of oil occurs during fruit development. When exactly oil accumu-lation starts in this tissue is still uncertain. Lubis (1983) reported that oil synthesis starts at three months after anthesis. Other publication stated that period of active oil synthesis begins at around 15 weeks after anthesis (WAA) (Abdullah *et al.*, 1994), whereas Budiani (2000) reported that the time when oil accumulation starts is different from one to another individual plant. One plant may start to synthesis oil at 16 WAA and other plant start at 19 WAA. These all data suggested that the fruit age or WAA can not be generalized as the time when exactly oil palm start the active period of oil biosynthesis.

Many enzymes involved in oil bio-synthesis, such as acetyl-CoA carboxylase (ACCase), acetyl-CoA:ACP transacylase, malonyl-CoA:ACP transacylase, β-ketoasil-ACP synthetase, β-ketoasil-ACP reductase, β-hidroksiacyl-ACP dehydratase, enoyl-ACP reductase, acyl-ACP thioesterase, and other enzymes that catalyze formation of triacyl glycerol (Lehninger, 1993). One of those enzymes that are considered to be a key enzymes for oil biosynthesis is ACCase. This enzyme catalysis formation of malonyl-CoA from acetyl-CoA, the first committed step in the *de novo* biosynthesis of fatty acid (Harwood & Page, 1994). Many publication showed that ACCase is a regulatory enzyme, and the reactions catalyzed is considered to be the rate limiting step on the fatty acid biosynthesis (Hablacher *et al*., 1993). Harwood & Page (1994) reported that changes in ACCase activity correlate well with lipid accumu-lation in seeds of castor been, rape and *cuphea*, indicating that this enzyme may have an important regulatory role in seed lipid biosynthesis.

It seems that certain enzymes involved in fatty acid biosynthesis in oil palm mesocarp are only or abundantly present at the period of oil synthesis while the regulatory proteins which are involved in switching on or increasing the level of expression of the genes coding for these enzymes may be present at the start or just before the period of active oil synthesis (Abdullah *et al.*, 1994).

As a part of research work on cloning of genes encoding key enzyme or regulatory proteins in palm oil biosynthesis, this research was aimed to isolate and identify protein differentially expressed during oil biosynthesis in the oil palm mesocarp.

Materials and Methods

Plant materials

Oil palm (*Elaeis guineensis,* Jacq.) of Tenera type inflorescences were tagged at anthesis and the fruits were harvested at different developmental stages (8, 10, 12, 15, 17, 19, 21, 22 and 24 WAA). After removal of the exocarp and kernel, the mesocarp tissues were put immediately in liquid nitrogen.

Analysis of oil content in the mesocarp

Mesocarp tissues were cut into pieces, weighed and dried in an oven at 70^0C for 16 hours. After weighing, the tissues were ground and weighed again. The sample was wrapped with filter paper, and put in a tube of soxhlet apparatus containing petroleum benzene. Oil extraction was carried out for about 8 hours. After 8 hours boiling or until solution turned clear (no more oil could be extracted), the flash was removed and dried, so that only oil remained in the flash. The flash was then weighed again. Oil weight was determined by subtracting the flash weight after and before soxhletisation.

Analysis of protein content in the mesocarp

Using coffee mill, mesocarp tissue was ground with liquid nitrogen and homogenized with extraction buffer. In the preliminary experiment, four different extraction buffers were tested, i.e. buffer A (100 mM Tris-HCl pH 8.2; 4 mM EDTA, 10 mM 2-mercaptoethanol), buffer B (0.1 M Tris-HCl pH 8.2; NaCl 0.5 M, EDTA 1 mM, β-mercaptoethanol 2%), buffer C (300 mM NaC l,1 mM EDTA, 2% ampholyte, 100 μg/mL leupeptin), and buffer D (300 mM NaCl, 1 mM EDTA, 2% ampholyte, 200 μg/mL leupeptin). Buffer that produces the best quality of SDS-PAGE pattern was chosen for protein Sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Identification of protein

In order to identify protein differentially expressed during fruit development, which might be used as a marker for oil biosynthesis activity in oil palm, SDS-PAGEextraction from different ages of palm mesocarp. The homogenate was then centrifuged at 20.000 g for 30 minutes at 2^0C and the supernatant was collected and kept at -20^0C.Protein was determined quantitatively by Lowry method (Deuscher, 1990) and qualitatively using Sodium Dodecyl and two-dimensional (2-D) electrophoresis were done. SDS-PAGE was carried out using the method of Andrew (1986) with 4.5% stacking gel and 12% separating gel at a constant voltage of 150 volt. Following electrophoresis, gel was stained with silver nitrate (Morissey, 1981), and the molecular weight (MW) of the protein was determined based on the standard curve of log MW versus Rf from the standard proteins.

For 2D-electrophoresis, protein was firstly applied to isoelectric focusing (IEF)

gel according to the method described by Robertson *et al*. (1987). The gel mixture consisted of 2.7 mL H_2O, 2.0 mL acrylamide (30% w/v acrylamide, 2.67% bisacrylamide), 2.4 mL 50% glycerol, 0.6 mL ampholyte (Biolyte, BioRad), 6 g. urea, 40 µL Triton X-100, 50 µL ammonium persulfate and 20 µL N,N,N',N'- Tetra-methylethylenediamine (TEMED). Electro-phoresis was performed at 150 volt constant voltage for 30 minutes, and then increased at 200 volt for 2.5 hours. The gel was stained with Coomassie Brilliant Blue and the lane (sample) of interest was excised and placed in direct contact with SDS-PAGE gel system for the second dimension. The two-dimensional gel was then stained with silver nitrate according to Morissey (1981).

Protein sequencing and BlastP analysis

Proteins of interest, i.e. proteins with with molecular weight 31.0 kDa and 34.3 kDa,that those expression increased coincide with oil content were isolated and purified from the gel and sent to Plant Reseach Institute, The Netherlands, for partial amino acid sequencing. The resulted polypeptides were subjected to BlastP for analyzing homology of the amino acid sequence with that of oil biosynthetic enzymes.

Results and Discussion

Effect of buffer composition

The result showed that for almost all of the buffers used, total protein increases as the oil content increases (Table 1). Buffer C and D produced higher total protein than other two buffers. This might be caused by

Table 1. Concentration of total protein extracted by four different buffers from three different bunches of palm fruits.

Tabel 1. Konsentrasi protein total terekstraksi dengan empat bufer berbeda dari tiga tandan kelapa sawit

Buffer type *Tipe bufer*	Fruit bunch *Tandan buah* no	Oil content *Kandungan minyak* (% g/fresh weight) (%/g *berat segar*)	Total protein *Protein total* (µg/g)
A	1	1.05	95.08
	2	2.41	97.71
	3	2.88	10.97
B	1	1.05	10.81
	2	2.41	11.52
	3	2.88	11.77
C	1	1.05	12.94
	2	2.41	12.90
	3	2.88	13.88
D	1	1.05	11.37
	2	2.41	13.46
	3	2.88	13.50

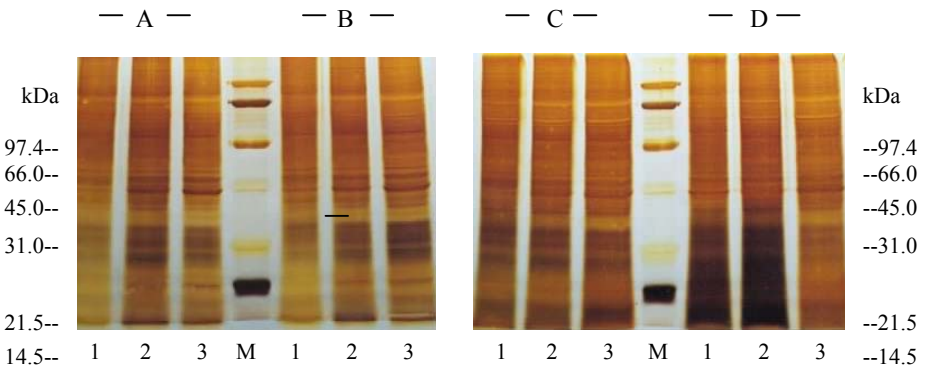

Figure 1. SDS-PAGE patterns of mesocarp proteins from three different fruit bunches (1-3) extracted by four different buffers (A-D). M : protein standard.

Gambar 1. Pola SDS-PAGE pada mesokarp protein dari tiga tandan buah yang berbeda (1-3) di ekstraksi dengan empat bufer yang berbeda (A-D). M: standard protein.

the presence of leupeptin, a protease inhibitor, in the two buffers.

SDS-PAGE pattern of the total protein showed that even no significant differences, buffer A and B produced patterns with more intense and discreet protein bands compared to that of protein bands from buffer C and D (Figure 1). Therefore buffer A was then used in the subsequent experiments.

Oil and protein content

Total protein and oil content in the mesocarp from different ages of fruits showed that oil content increased sharply in the fruit of 19 WAA (23.04% from 1.78% at 17 WAA) (Figure 2). From other experiment using different plant of oil palm, it was found that at the 18 WAA the oil content was 16.01% (data is not shown). These data reflect that the period of active oil synthesis might be started at 17 WAA. However, in contrast to the data showed that no correlation between concentrations of total protein with oil content or developmental stage of the fruit (Table 2, Figure 2.)

Identification of protein

SDS-PAGE patterns of protein from different ages of palm mesocarps clearly shown that the expression of 2 proteins increased sharply before (34.3 kDa-protein) and at the beginning (31.0 kDa-protein) of active oil biosynthesis period (Figure 3). The 34.3 kDa-protein was present initially at 8 – 12 WAA in very low intensity, but then its expression sharply increased at 15 WAA. Another protein, with MW 31.0 kDa protein, was also weakly expressed at 8 – 15 WAA and then highly expressed at 17 WAA. These data show that both proteins were differentially expressed during mesocarp development, and were highly expressed just before the initiation period of active oil synthesis started. Therefore it was predicted that these proteins might be part of regulatory protein or key enzyme in oil biosynthesis and potentially used as marker

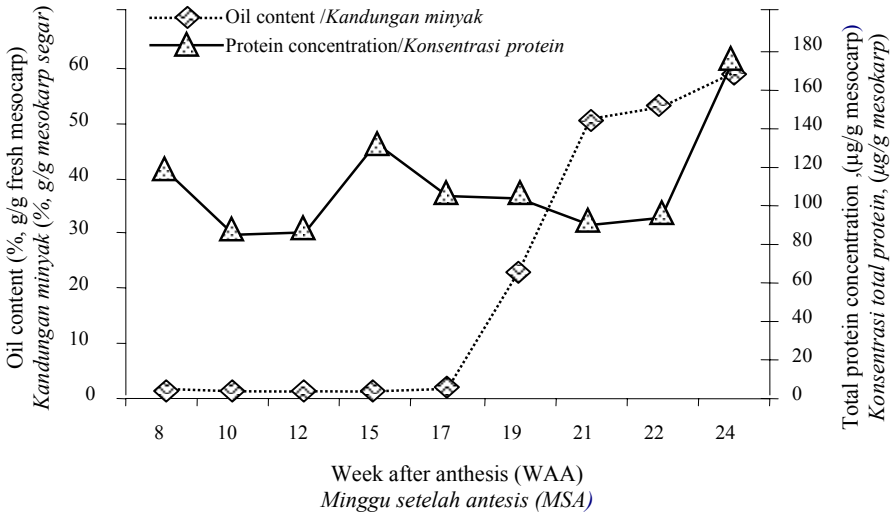

Figure 2. Oil and protein content of oil palm mesocarp from different developmental stages (8 – 24 WAA).

Gambar 2. Kandungan minyak dan protein pada mesokarp kelapa sawit dari tahap perkembangan yang berbeda (8-24 MSA)

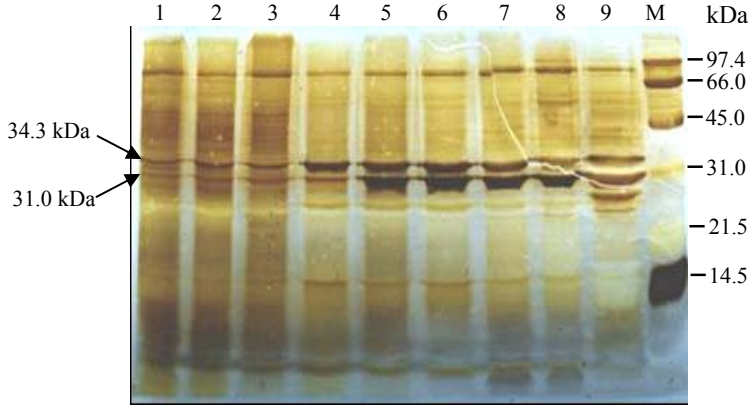

Figure 3. SDS-PAGE pattern of protein from different ages of palm mesocarps (M : standard protein; (1)8 WAA, (2)10 WAA, (3)12 WAA, (4)15 WAA, (5)17 WAA, (6)19 WAA, (7)21 WAA, (8)22 WAA, (9)24 WAA.

Gambar 3. Pola SDS-PAGE dari protein dengan umur mesokarp yang berbeda (M: protein standard; (1)8 MSA, (2)10 MSA, (3)12 MSA, (4)15 MSA, (5)17 MSA, (6)19 MSA, (7)21 MSA, (8)22 MSA, (9)24 MSA.

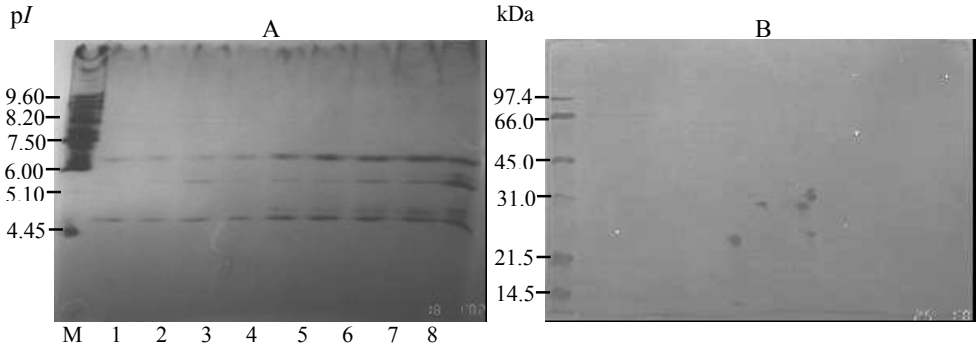

Figure 4. IEF pattern of mesocarp proteins from different ages of seeds (1)8 weeks, (2)10 weeks, (3)12 weeks, (4)15 weeks, (5)17 weeks, (6)19 weeks, (7)21 weeks, (8)22 weeks, (9)24 weeks.

Gambar 4. Pola IEF protein dari mesokarp dengan umur benih yang berbeda (1)8 minggu, (2)10 minggu, (3)12 minggu, (4)15 minggu, (5)17 minggu, (6)19 minggu, (7)21 minggu, (8)22 minggu, (9)24 minggu.

Table 2**.** Distribution and characterization of protein bands from different ages of oil palm mesocarps.

Tabel 2. Distribusi dan karakterisasi pita protein dari mesokarp kelapa sawit dengan umur yang berbeda

Fruit Age (week) *Umur buah* (*minggu*)	Protein band/ *Pita protein*		Band intensity *Intensitas pita*
	p*I*	MW (kDa)	
8	4.56	34.3	+
	4.56	22.8	+
	5.88	22.8	+
17	4.56	34.3	++
	4.56	22.8	++
	4.64	31.0	++
	4.95	31.0	++
	5.88	22.8	++
22	4.56	34.3	+++
	4.56	23.3	++
	4.56	14.5	+
	4.64	31.0	+++
	4.95	31.0	+++
	5.88	23.3	++
	5.88	14.5	+

proteins for oil biosynthesis in oil palm mesocarp. For further analysis, both proteins were isolated and purified from the gel using Model 422 Electro-Eluter (BioRad).

From the IEF analysis, it was shown that several proteins with pI 5.88; 4.56; 4.95, and 4.64 increased in certain developmental stages of mesocarp (Figure 4). The proteins of pI 5.88 and pI 4.56 were detected in very low intensity at the 8 WAA and become more intense as the fruit age increases. Other proteins with pI 4.95 and pI 4.64 were present firstly at the 17 WAA, and then become more intense at the older mesocarp.

Three lanes of sample, 8 WAA, 17 WAA, and 22 WAA, were cut from the IEF gel and applied to the SDS-PAGE for the second dimension. The results show that at the palm mesocarp of 8 WAA, two weak protein bands (34.3 and 22.8 kDa) with the same pI 4.56 and a protein with MW 22.8 kDa with pI 5.88 were detected (Figure 4). Two more intense protein bands (34.3 kDa and 22.8 kDa) with pI 4.56, two protein ands of the same MW (31 kDa) with pI 4.64 and 4.95, and another protein (22.8 kDa) with pI 5.88 were found at the 17 WAA. At the 22 WAA several more intense protein bands were identified. At 22 WAA, three proteins (34.3, 23.3 and 14.5 kDa) were found at pI 4.56, two proteins of 31 kDa at pI 4.64, and 4.95, and other two proteins at the same pI 5.88 (23.3 and 14.5 kDa) (Table 2). These 2-D electrophoresis results show that protein of 31.0 kDa at least composed of two polypeptides with pI 4.64 and 4.95, whereas protein of 34.3 kDa is a single protein with pI 4.56

BlastP analysis of the polypeptides resulted from partial sequencing of both proteins show some homology between the two proteins with enzymes involved in oil biosynthesis. Two polypeptides out of 91 polypeptides derived from sequencing of 31.0 kDa protein were found having homologous with biotin carboxylase subunit of Acetyl-CoA Carboxylase (ACCase) and four polypeptides homologous with enoyl–ACP reductase. Five polypeptides out of 81 from 34.3 kD protein matched with glyceraldehyde-3 phosphate dehydrogenase (Table 3).

ACCase is an enzyme directing the carbon flow from photosynthesis to primary and secondary metabolites in plant. This enzyme catalyzes formation of malonyl-CoA from acetyl-CoA and bicarbonate, the first committed step in oil biosynthesis pathway.

This reaction is considered to be the rate limiting step in fatty acid biosynthesis and oil accumulation in several plants (Page *et al*., 1994; Shintani & Ohlrogge, 1995; Heith & Rock 1995). Two forms of ACCase have been identified in plants, heteromeric and homomeric forms (Li & Cronan 992; Sasaki *et al.* 1993, 1995; Alban *et al*. 1994; Shorrosh *et al*. 1994, 1995). The heteromeric ACCase (ht-ACCase) consist of four dissosiable proteins or subunits; namely biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), carboxyl transferase α and β (α- and β-CT).

Enoyl-ACP reductase (ENR) is a component of fatty acid synthase, that catalyzes the final step in condensation reaction of malonyl-CoA to form acyl-ACP. This reaction is considered to be one of key regulatory steps in the elongationof fatty acid (Heith & Rock 1995; Bergler *et al*., 1996; Heith *et al*. 2000). Fawcett *et al*., (1994) reported that steady-state level of ENR transcript in *B. napus* increased during lipid deposition in seed and the optimum mRNA expression preceded the maximum level of the protein, which in turn preceded the maximum level of oil. Analysis of immunoassay in rape seed demonstrated that the enzyme continually increase during lipid deposition (Slabas *et al.,* 1990). An amino acid substitution in ENR of *A. thaliana* mutant causes a marked decrease in its enzymatic activity, impairing fatty acid biosynthesis and decreasing the amount of total lipid, causes premature cell death (Mou *et al*., 2000). These all publications indicated that ENR

Table 3. Peptide sequence of the 31.0 kDa and 34.3 kDa protein and their homologous search analysis.

Tabel 3. Sekuens peptida dari protein 31.0 kDa dan 34,3 kDa dengan analisis homologus

Protein band *Pita protein*	Peptide sequence *Sekuens peptida*	Homology to enzyme *Enzim homologi*
31 kDa	AVLEEAPSPALTPELR QLFVDEDPLPTPELR	ACCase, s.u. biotin carboxylase
	MLEYSYANAPLAGGK QALETQGLAQAFEAGR QALESDTFVLAFEAGR SGGLKCEVVSGLPLDLR	Enoyl-ACP-reductase
34 kDa	NGLLGYYESVNVALTFLGDSR QLSPLVYQEVVATLDPFLDAK VPTVDVSVVDLTVR GLGLNGFGR RPTVDVSVVPAFLR	Glyceraldehyde-3 phosphate dehydrogenase

play an important role in fatty acid and oil bio-synthesis. Gliseraldehyde-3P-dehydro-genase is one of enzymes involved in glycolysis, a pathway that convert glucose into pyruvate. This enzyme catalyzes formation of 1,3 biphosphoglycerate (1,3-BPG) from glycer-aldehyde-3-phosphate (GAP). Pyruvate is then further converted into acetyl-CoA, a precursor of many metabolic pathways, one of them is fatty acid synthesis. Francki *et al*. (2002) identified gene differentially expressed during lipid accumulation in lupin species by differential display (DDRT). DNA analysis of the gene partially cloned showed similarity with the family of glucose dehydrogenase reported in barley.

The homology of the detected proteins differentially expressed coincide with oil accumulation in oil palm mesocarp with three different enzymes, ACCase subunit BC, ENR and Glyceraldehyde-3P dehydro-genas generate a further question: whether all the enzymes involved directly (ACCase, FAS and enzymes for synthesis of triacylglycerol) and indirectly such as enzymes for glycolysis are co-ordinately expressed and regulated during oil accumulation. A series of research will be needed to answer this question. On the other hand the information about polypeptide highly homologe with enzymes involved in oil biosynthesis, ACCase subunit BC and ENR found in this research have been used for designing degenerate primers in order to clone the genes encoding those enzymes.

Conclusion

Proteins with MW 31.0 kDa and 34.3 kDa were expressed differentially coincided with oil accumulation in the oil palm mesocarp. Expression of these two proteins increased sharply at the beginning and just before the period of active oil biosynthesis respectively. The 31.0 kDa-protein consist of two proteins, with pI 4.64 and pI 4.95, whereas 34.3 kDa-protein is a single protein with pI 4.56. Partial amino acid sequencing data of the 31.0 kDa protein

showed that two polypeptides highly homologous with ht-ACCase biotin carboxylase subunit, and four polypeptides homologous with enoyl-ACP reductase, whereas the 34.3-kDa protein showed homology with glycerol-dehyde-3 phosphate dehydrogenase.

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