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BIOCHEMICAL STUDIES AND PURIFICATION OF OIL PALM (*Elaeis guineensis* Jacq.) β-KETOACYL-ACYL-CARRIER-PROTEIN (ACP) SYNTHASE (KAS) II ENZYME

UMI SALAMAH RAMLI*; MOHAMMED NOOR EMBI; OTHMAN OMAR** and RAVIGADEVI SAMBANTHAMURTHI*;**

ABSTRACT

*The rapid increase in palm oil production over the last 20 years has made this oil the most important in the world. Palm oil produced in the mesocarp of the oil palm (*Elaeis guineensis*) fruit contains primarily palmitic (44%) and oleic (39%) acids with only a small proportion of polyunsaturated fatty acids. As a prelude to detailed biochemical analysis of the fatty acid biosynthesis of oil palm, we examined β-ketoacyl-ACP synthase (KAS) II activity, and evidence is presented on its importance in palmitic acid accumulation. In this study, three different oil palm breeding materials were screened for KAS II and fatty acid compositions. Collectively, the results strongly suggest that KAS II plays an important role in determining the relative amounts of C16 and C18 fatty acids in oil palm. KAS II activity was profiled through various stages of fruit development from 12 to 22 weeks after anthesis (WAA). The data depicted a pattern of progressive increase in KAS II activity similar to that of triacylglycerol synthesis in the oil palm mesocarp which started at 15 WAA and reached a maximum around 20 WAA. The enzyme was purified to homogeneity (>9000-fold purification) using a combination of ion exchange on CM-Sepharose, HR-DEAE, hyroxyapatite and affinity chromatography (ACP-Sepharose). Activity of the purified enzyme was inhibited by the chelating agent ethylene diamine tetra-acetate (EDTA) (1 M), but the inhibitory action could be overcome by divalent cations such as Mn^{2+} or Mg^{2+} (10 mM). Optimal activity was observed at pH 4.5. The data represent the first contribution to the biochemical characterisation of oil palm KAS II activity associated with fatty acid biosynthesis.*

Keywords: oil palm (*Elaeis guineensis*), β-ketoacyl-acyl-carrier-protein (ACP) synthase (KAS) II, enzyme purification, fatty acid biosynthesis.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is now the world's most important edible oil when ranked by global production and consumption. Malaysia and Indonesia produce most of the world's palm oil, accounting for approximately 86% of total production. The mesocarp of the ripe oil palm fruit accumulates large amounts of oil which contains palmitic acid (C16:0) at up to 44% of the total fatty acids. Other major fatty acids in palm oil include oleic and stearic acids which account for 39% and up to 5% of the total composition, respectively (Tan and Oh, 1981). Palm oil tends to feed into relatively low-value generic commodity streams, whether for food or oleochemical use (Murphy, 2009). New palm oil tailored for specific markets would thus have value-addition and would compete more effectively to meet the demands from the expanding oil business and changing market requirements.

* Malaysian Palm Oil Board, P. O. Box 10620, 50720 Kuala Lumpur, Malaysia.
E-mail: umi@mpob.gov.my
** Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43650 UKM Bangi, Selangor, Malaysia.

Biotechnology applied to oils and fats has potential to exploit the opportunities in creating oil crops with dramatically altered lipid composition (Murphy, 1994). Vegetable oil stability and quality are primarily determined by its fatty acid composition, especially the relative proportion of saturated and unsaturated fatty acids. There is little available information on the biochemical analysis of the enzyme activities in oil palm and on the molecular mechanisms that control the fatty acid composition of palm oil. An understanding of the biochemical factors and metabolic pathways regulating oil composition is necessary for exploiting opportunities and tailoring the palm oil for use in manufacturing value-added products. For that reason, we studied the biochemical factors affecting oil composition (Ramli and Sambathamurthi, 1996; Ramli *et al*., 2002a, b; 2009) as part of an initiative to modify the fatty acid composition of palm oil (Parveez *et al*., 2009).

The β-Ketoacyl-ACP synthase (KAS) II is a component of the Type II fatty acid synthase (FAS) located in plastids (Harwood, 1988). Plant FAS contain four β-ketoacyl-ACP synthases; KAS I, KAS II, KAS III and KAS IV, with different substrate specificities (Harwood, 1988; Dehesh *et al*., 1995). KAS I catalyses the condensation of malonyl-ACP with acyl-ACP primers of between 2 and 14 carbons and, thus, produces palmitate as its longest chain product. KAS II catalyses the production of stearate from palmitate. Both KAS I and KASII can also be differentiated on the basis of their sensitivity to inhibitors such as cerulenin and arsenite (Harwood, 1988; 1996). The short-chain condensing enzyme KAS III was discovered in plants (Jaworski *et al*., 1989); it catalyses the initial reactions of FAS (Walsh *et al*., 1990). A cDNA clone isolated from *Cuphea* sp. has been designated to encode a novel KAS IV which has a different acyl-chain specificity to the previously characterised KAS (Dehesh *et al*., 1995).

The first purification and demonstration of the roles of KAS I and KAS II from plants were achieved in the 1980s using spinach (Shimakata and Stumpf, 1982), barley (Hoj and Mikkelsen, 1982), parsley (Schuz *et al*., 1982) and rapeseed (MacKintosh *et al*., 1989). Up until now, only little information on the purification of KAS II enzymes from plants is available. Therefore, this investigation reports on: (i) the screening of KAS II activity from various oil palm breeding materials with different fatty acid compositions to determine the role of KAS II in fatty acid biosynthesis, (ii) a study into the KAS II activity profile in developing oil palm fruits, and (iii) the first purification and characterisation of oil palm KAS II. We used the method described by MacKintosh *et al*. (1989) to investigate KAS II activity and achieved a purification of near homogeneity from the oil palm fruit mesocarp. Our data suggest that KAS II plays an important role in controlling the C16:C18 ratio of palm oil fatty acids, consistent with proposals for other crops (Harwood, 1996).

MATERIALS AND METHODS

Plant Materials

Oil palm (*Elaeis guineensis* var. *Tenera*) fruits were obtained at 18-19 weeks after anthesis (WAA) from the MPOB Research Station, Bangi, Selangor, Malaysia. The *tenera* variety was used for enzyme assay, determination of iodine value (IV), fatty acid analysis and enzyme purification. Fruits of the other oil palm species, *Elaeis oleifera*, and of its hybrids with *E. guineensis*, were obtained from the MPOB Research Station, Kluang, Johor, Malaysia, and were used for determination of IV, fatty acid analysis and enzyme assay.

Radioactive Materials and Chemicals

Sodium [^{14}C] bicarbonate (51.8 mCi mmol^{-1}) was purchased from Amersham International (Amersham, United Kingdom). CM-Sepharose and hydroxylapatite were sourced from BioRad (United Kingdom). Diethylaminoethyl cellulose was from Whatman Biosystem (United Kingdom), while HR-DEAE-cellulose was purchased from Waters Biochemicals (United Kingdom). All other reagents were of the highest purity available from Pharmacia, BioRad or Sigma.

Purification of *E. coli* Acyl Carrier Protein

Acyl-carrier protein (ACP) was prepared from *E. coli* (Grain Processing, Muscatine, IA) as described by Rock and Cronan (1980). The *E. coli* cells were broken using a microfluidiser at 16 000 psi. The ACP preparation was frozen at -70°C until required.

Preparation of *E. coli* Fraction A

Fraction A which comprises three *E. coli* fatty acid synthase enzymes (acetyl CoA:ACP transacylase, malonyl CoA:ACP transacylase and β-ketoacyl ACP synthase II) was prepared according to Majerus *et al*. (1969). The preparation was then cerulenin-treated as described by MacKintosh *et al*. (1989). The cerulenin-treated fraction A was frozen in 0.5 ml aliquots at -70°C.

KAS II Assay

KAS II activity was assayed as described by MacKintosh *et al.* (1989) with some modifications. The assay mix (total volume of 0.25 ml) contained

50 mM acetate (pH 4.5), 2 mM dithiothreitol, 20 mM sodium bicarbonate, 10 μg cerulenin-treated fraction A, 10 μg ACP from *E. coli*, 5 μM malonyl CoA, 30 μM stearoyl CoA and 26 μM sodium [^{14}C]-bicarbonate (51.8 mCi mmol^{-1}). The reaction was initiated by the addition of mesocarp extract and incubated at 30^{o}C for 10 min. For the determination of KAS II activity at the various stages of fruit development, a mesocarp extract prepared from different stages of oil palm development was added into the assay. The reaction was terminated by adding 40 μl of 10% perchloric acid. Following centrifugation at 13 000 rpm in an eppendorf rotor for 5 min, the supernatant was removed into a scintillation vial and dried in a vacuum concentrator. Ten millilitres of optifluor scintillant (Canberra Packard) were added to the dried assay sample and radioactivity determined using an LKB Wallac scintillation counter (Perkin-Elmer). Assays were carried out together with a blank assay which contained no mesocarp extract. One unit of KAS II activity was defined as 1 nmol of [^{14}C]malonyl CoA formed per minute derived from the reverse reaction. Data points represent the averages of triplicates within an experiment. For experiments on the effect of sulphydryl reagents and metallic ions, the enzyme was pre-incubated for 10 min at 30^{o}C with each compound.

Protein Determination

Protein was measured by the method of Lowry *et al.* (1951). Calibration curves were constructed from a standard stock solution of bovine serum albumin.

Determination of Iodine Value and Fatty Acids

The iodine value (IV) and fatty acid composition of the oil palm samples were determined according to PORIM Test Methods (PORIM, 1995).

Purification of KAS II

All five steps involved in the purification of KAS II were carried out at 4^{o}C unless otherwise stated.

Preparation of acetone powder extract. Oil palm acetone powder extract was made by homogenising fresh mesocarp with five times its weight of ice-cold acetone. After standing at 4^{o}C for approximately 30 min, the homogenate was filtered through a high speed vacuum pump (Pharmacia LKB). The powder was stored at -80^{o}C. Approximately 50 g of mesocarp acetone powder were ground in 250 ml buffer containing 0.1 M potassium phosphate (pH 7.0), 2 mM dithiothreitol, 1 μM leupeptin, 1 μM pepstatin and 0.1 μM polymethylsulfonylfluoride. The homogenate was filtered through a layer of muslin cloth and centrifuged at 18 000X g for 15 min. The supernatant was decanted through glass wool to remove the fat layer. PVPP was added to the supernatant and centrifuged to sediment PVPP. The final supernatant was concentrated to approximately 150 ml on an Amicon Hollow fibre cartridge HIP30-43 (Amicon, USA), and dialysed against 20 mM potassium buffer, pH 6.5.

Purification by carboxymethyl (CM)-Sepharose chromatography. A 3 x 40 cm column of CM-Sepharose (Pharmacia) was equilibrated with 0.02 M potassium phosphate (pH 6.5). The dialysed preparation was loaded into the column and washed with the same buffer until absorption of the effluent at 280 nm returned to baseline. The flow rate throughout was 2 ml min^{-1}. The effluent (which contained the KAS II activity) was pooled.

Purification by HR-DEAE cellulose chromatography. The CM-Sepharose elution pool (approximately 100 ml) was applied to a 2 x 20 cm HR-DEAE-cellulose column equilibrated with 0.02 M potassium phosphate (pH 6.5). The column was washed with the buffer until absorption at 280 nm returned to baseline, and the enzyme was eluted using a linear gradient of 0-1 M NaCl in 0.1 M potassium phosphate buffer (pH 6.5). The flow rate throughout was 2 ml min^{-1}. The active fractions were concentrated to ~ 20 ml on an Amicon PM-30 membrane concentrator, and dialysed against 0.02 M potassium phosphate (pH 6.5).

Purification by hydroxyapatite chromatography. The dialysed sample was loaded onto a hydroxyapatite column (2 x 10 cm) equilibrated with 0.02 M potassium phosphate (pH 6.5). The column was washed with 0.1 M potassium phosphate (pH 6.5) and the enzyme eluted in 0.4 M potassium phosphate (pH 6.5). The flow rate throughout was 0.1 ml min^{-1}. The fractions containing high KAS II activity were pooled, concentrated for 90-120 min by dialysis against 0.02 M potassium phosphate (pH 6.5).

Purification by ACP-Sepharose affinity chromatography. The pooled concentrate (approximately 4 ml) was loaded into an ACP-Sepharose column (1.5 x 6.0 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 6.5). The column was prepared according to McKeon and Stumpf (1982) using *E. coli* ACP (approximately 25 g ml^{-1}) as ligand. Following the loading of the sample, the column was washed with equilibration buffer and eluted with 0.1 M potassium phosphate buffer (pH 6.5). The flow rate throughout was 0.1 ml min^{-1}. The fractions containing KAS II activity were pooled and stored at -70^{o}C.

Polyacrylamide (PAA) Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed on a 5%-20% gradient using a 1.5 mm thick gel and the Laemmli (1970) discontinuous buffer system. The relative molecular mass of KAS II was determined from its mobility relative to those of standard proteins ranging from 14 to 181 kDA (Sigma).

RESULTS

Screening of KAS II Activity from Various Oil Palm Breeding Materials and Correlation with Fatty Acid Composition

KAS II activity was investigated in three groups of oil palm breeding materials: *E. oleifera*, *E. guineensis* and their hybrids. They had IV varying from ~55 to ~90, reflecting the unsaturated fatty acid contents of their oils (*Table 1*). IV determined in palm oil measures the number of double bonds in the oil, and is used as a parameter in process control as well as a quality parameter in traded palm oil products (PORIM, 1995). Palm oil obtained from the mesocarp of *E. guineensis* contains 44% of palmitic acid, while in the South American oil palm species, *E. oleifera*, the level of oleic acid is much higher (close to 70%) with a lower amount of palmitic acid C16:0 (25%) (Rajanaidu *et al*., 2000). Palms from each population were selected and screened for KAS II activity, IV and fatty acid composition (*Table 1a*). The different populations showed wide ranges for the major fatty acids: pamitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3). The data were then subjected to a series of statistical analysis to investigate whether enzyme activity could be used to distinguish the three different populations, and to compare the correlation network with fatty acid composition. Visual inspection revealed clustering of KAS II activity according to the populations. Significant differences in KAS II activity were observed with the *E. oleifera* lines which showed the highest KAS II activity, followed by the hybrids and then *E. guineensis* ($p<0.001$) (*Table 1b*). Regression analysis uncovered a significant negative correlation ($P<0.001$) between C16:0 content and KAS II activity. In contrast, KAS II activity was positively correlated with IV and C18:1 as well as the total saturated and unsaturated C18 fatty acids (C18:0 + C18:1 + C18:2 + C18:3). However, KAS II activity had little effect on C18:3, thus indicating that the strong positive correlation observed for KAS II *vs.* (C18:0 + C18:1 + C18:2 + C18:3) was actually

TABLE 1a. KAS II ACTIVITY, IODINE VALUE* AND FATTY ACID COMPOSITIONS OF SELECTED THREE OIL PALM POPULATIONS (*Elaeis guineensis*[1], hybrids[2] and *Elaeis oleifera*[3])

Palm No.	Fatty acid composition (% total fatty acids)							
	KAS II activity[c] (nmol/mg/10 min)	Iodine value (IV)	C16:0	C18:0	C18:1	C18:2	C18:3	C18:1+C18:2 +C18:3
0.151/1280[1]	2.2	60.8	30.8	6.6	49.0	11.7	0.5	61.2
0.151/1885[1]	1.9	57.3	36.5	4.5	50.3	8.0	0.2	58.5
0.151/1207[1]	1.2	57.8	36.1	7.4	43.9	11.7	0.3	55.9
0.151/1774[1]	1.0	54.6	38.4	5.6	46.3	7.8	0.9	55.1
0.151/1787[1]	0.5	50.1	39.8	4.0	47.7	7.6	0.2	55.5
Mean ± S.D[a]	1.3 ± 0.7	56.1 ± 4.0	36.3 ± 3.4	5.6 ±1.4	47.4 ± 2.5	9.4 ± 2.1	0.5 ± 0.3	57.2 ± 2.6
0.177/46[2]	3.9	67.8	24.1	n.d.	n.d.	n.d.	n.d.	n.d.
0.177/28[2]	3.9	64.1	24.0	2.6	65.7	5.9	0.8	71.6
0.177/43[2]	3.4	63.8	22.2	2.6	69.2	5.4	0.4	75.0
0.177/41[2]	2.3	62.1	26.9	2.3	63.9	5.4	0.6	69.9
Mean ± S.D[b]	3.4 ± 0.8	63.3 ± 2.4	24.3 ± 1.9	2.5 ± 0.2	66.3 ± 2.7	5.6 ± 0.3	0.6 ± 0.2	72.2 ± 2.6
0.211/1572[3]	7.5	88.5	17.7	0.8	56.8	22.7	1.1	80.6
0.211/1280[3]	6.9	85.8	13.4	0.9	68.7	15.2	1.1	85.0
0.211/1646[3]	5.6	84.3	16.4	0.9	61.8	18.7	1.0	81.5
0.211/1496[3]	5.3	83.4	15.0	0.9	59.9	21.5	1.3	82.7
0.211/1577[3]	4.1	84.0	18.9	1.3	60.2	17.3	1.0	78.5
Mean ± S.D[a]	5.9 ± 1.3	85.2 ± 2.1	16.3 ± 2.2	1.0 ± 0.2	61.5 ± 4.4	19.1 ± 3.1	1.1 ± 0.1	81.5 ± 2.5

Note: Each individual sample was measured in triplicate.
[a]Means ± s.d. (n = 5).
[b]Means ± s.d. (n = 4).
[c]Significantly different ($p<0.001$) between *Elaeis guineensis*, *Elaeis oleifera* and hybrids.
*Iodine value was determined according to PORIM Test Method (1995).

TABLE 1b. REGRESSION ANALYSIS OF RELATIONSHIP BETWEEN KAS II ACTIVITY, PALM OIL FATTY ACID COMPOSITION AND IODINE VALUE (IV)

Variable	Correlation coefficient	Regression coefficient	P
KAS II *vs*. IV	0.94151	0.156525	<0.001
KAS II *vs*. C16:0	0.92004	-0.208826	<0.001
KAS II *vs*. C18:0	0.78831	-0.815512	<0.01
KAS II *vs*. C18:1	0.66193	-0.151869	<0.01
KAS II *vs*. C18:2	0.69005	-0.259508	<0.01
KAS II *vs*. C18:3	0.76302	4.220126	<0.01
KAS II *vs*. (C18:1 + C18:2 + C18:3)	0.92004	-0.174740	<0.001
KAS II *vs*. C18:1 and C18:2	0.95030	0.149917 (for 18:1)	<0.001
		0.256442 (for 18:2)	

Note: Regression analysis was performed using data from *Table 1a*.

contributed by C18:1 and C18:2. The C16:C18 ratio (calculated as C16:0/[C18:0 + 18:1 + 18:2 + 18:3]) was negatively correlated with KAS II activity and in the high oleate materials. The data reflect the role of KAS II in determining the ratio of C16:C18 fatty acids, and is thus consistent with findings in other crops (Aghoram *et al*., 2006; Pidkowich *et al*., 2007).

KAS II Activity during Fruit Development

To determine the optimum level of KAS II activity for enzyme purification, KAS II activity was screened in developing oil palm fruits. To coincide with periods of significant oil accumulation, oil palm fruits, between 12 and 22 WAA, were harvested and three replicate samples were used to minimise variations due to the developmental stage of the fruits and possible environmental factors impacting these measurements. The highest KAS II activity was observed in samples extracted from fruits of between 19 and 21 WAA, after which the activity declined (*Figure 1*). Accordingly, oil palm fruits between 19 and 21 WAA were used in further KAS II activity assays and in the preparation of the protein. The activities thus match the period of maximal oil accumulation in the mesocarp (Oo *et al*., 1985).

Purification of KAS II Enzyme from Oil Palm Mesocarp and Biochemical Characterisation

A summary of the purification steps for oil palm KAS II is given in *Table 2*. Several extraction procedures were attempted to purify KAS II from oil palm mesocarp in order to obtain the best yield and activity. Protein precipitation was first employed using ammonium sulphate. However,

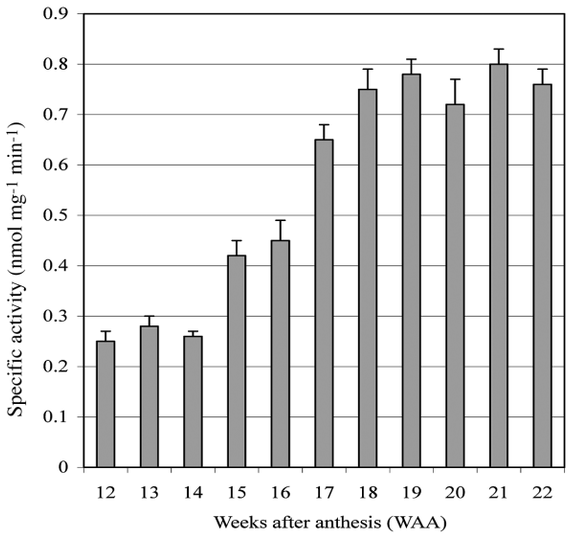

Figure 1. KAS II activity at various stages of fruit development. Oil palm inflorescences were tagged and fruits were freshly harvested at various stages. Enzyme activities were assayed as described under Materials and Methods. Means ± S.D. (n = 3).

this method proved unacceptable as low activity was observed in all fractions and almost a 70% loss of activity was detected. Another method tested was the addition of organic solvents (with low dielectric constants) such as cold acetone to discourage the dispersion of protein molecules in the mesocarp extract. Acetone precipitation had the advantage of removing lipid contaminants from the samples that could interfere with later purification steps. The method worked efficiently in concentrating the protein with a minimum loss of activity (less than 5%). Therefore, acetone precipitation was used in the subsequent crude extract preparation. A conventional ion-exchange chromatography

TABLE 2. THE PURIFICATION OF OIL PALM KAS II

Step	Total protein (mg)	Total activity (nmoles min^{-1})	Specific activity (nmoles mg^{-1} ml^{-1})	Purification factor	*Recovery (%)
Crude extract	800	60.5	0.08	1	100
CM-Sepharose	196	49.0	0.27	3	81
HR-DEAE	15	22.3	1.49	20	28
Hydroxyapatite	3	16.2	5.41	725	27
ACP-Sepharose	0.007	5.0	712.45	9 424	8.3

Note: *Recovery from pooled peak fractions.

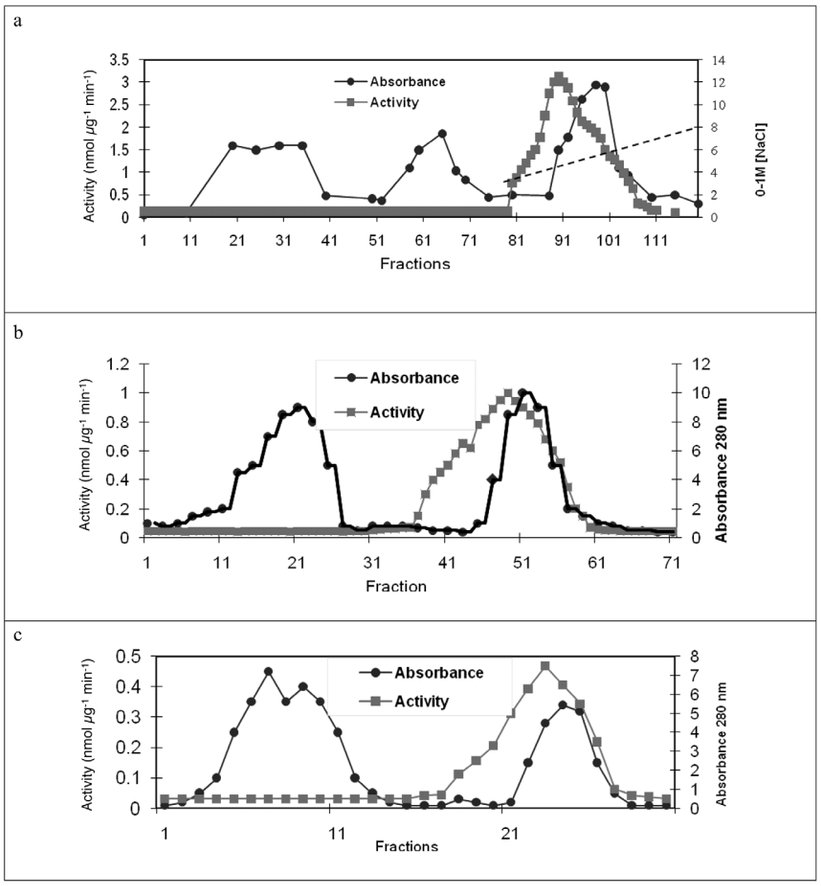

Figure 2. Purification of oil palm KAS II. Elution profile of KAS II on: (a) HR-DEAE column, (b) hydroxylapatite column and (c) ACP-Sepharose column as described under Materials and Methods.

was applied to remove positively charged proteins from the crude extract using CM-Sepharose. The first step of chromatography used CM-Sepharose chromatography of the crude acetone powder extract (see Materials and Methods). More than 80% of the recoverable activity was found in the flow-through of the column. In the second step, an anion-exchange column was used. Oil palm proteins were strongly bound to the HR-DEAE anion-exchange column even at an acidic pH (*Figure 2a*). A two-step gradient, *i.e.* 0.02-0.1 M potassium phosphate (pH 6.5) followed by 0-1 M NaCl in 0.1 M potassium phosphate (pH 6.5) was used to elute the proteins from the column. Purification of the crude enzyme by HR-DEAE column chromatography gave ~15-fold increase in purity with 27% recovery. An additional ~50-fold enrichment of KAS II activity was obtained by hydroxyapatite chromatography (*Figure 2b*). This was comparable to the roughly 34-fold enrichment reported by MacKintosh *et al.* (1989) for the oilseed rape KAS I using a similar hydroxyapatite step. The hydroxyapatite step was also essential for the protein to bind to the subsequent ACP-Sepharose column (*Figure 2c*) which greatly increased the purity of the enzyme (to 9000-fold with a yield of 8%) (*Table 2*).

The purified KAS II was run on a 5% polyacrylamide gel containing Pharmalyte 2.5-5.0

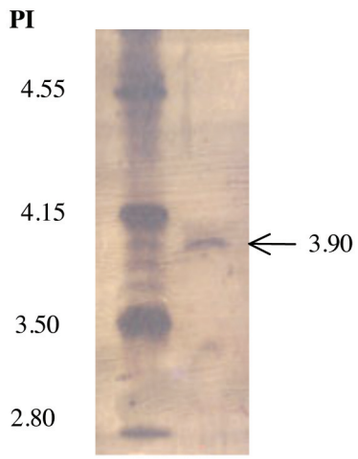

Figure 3. Isoelectric gel focusing on purified KAS II. Purified oil palm KAS II was loaded into SDS-polyacrylamide gel and subjected to isoelectric focusing as described under Materials and Methods. The gel was analysed on a polyacrylamide gel containing Pharmalyte 2.5 – 5.0.

and when subjected to isoelectric focusing, with the purified oil palm KAS II focused to a single major polypeptide having an isoelectric point (pI) of 3.9 (*Figure 3*). Upon electrophoresis in SDS-polyacrylamide, the purified KAS II migrated as a single band having an approximate Mr-value of ~48 000 Da (data not shown). Oil palm KAS II activity was apparently linear for 10 min and increased for 30 min before leveling off (*Figure 4*), and an optimal activity was recorded at pH 4.5 (*Figure 5*). The Ca^{2+} did not significantly influence KAS II activity (*Figure 6*). On the other hand, KAS II activity increased by 3- to 10-fold in the presence of Zn^{2+}, Mg^{2+} and Mn^{2+}, and was inhibited by 10 mM Cu^{2+}. The enzyme was 70% inhibited by 1 mm EDTA (*Figure 7*), but the inhibitory action of this chelating agent could be overcome by 10 mM Mg^{2+} or Mn^{2+}.

DISCUSSION AND CONCLUSION

Information on the relationship between KAS II enzymatic activity and fatty acid composition of plants has scarcely been reported. The role of KAS II in the overall regulation of the fatty acid biosynthetic pathway was examined by mutation studies in *Arabidopsis* (Carlsson *et al*., 2002) and soyabean seeds (Aghoram *et al*., 2006). These demonstrated a direct association between KAS II activity and palmitate levels which is consistent with our understanding of the biochemistry of fatty acid biosynthesis in plants. Furthermore, in another genetically manipulated study, the expression of FAB1 constructs in *Arabidopsis* demonstrates that reducing KAS II expression in a tissue-specific fashion provides an effective strategy for increasing 16-carbon fatty acid accumulation (Pidkowich *et al*., 2007). In fact, it was reported that a decrease in C16:0

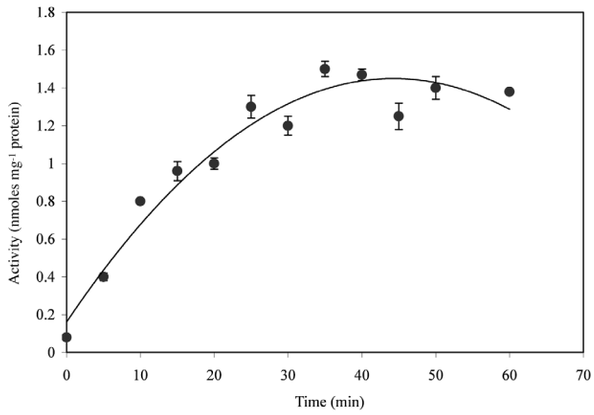

Figure 4. Effect of incubation time on KAS II activity. The purified enzyme was incubated under standard conditions for various periods. At the time specified, the reaction was stopped by adding 40% perchloric acid. Means ± S.D. (n=3).

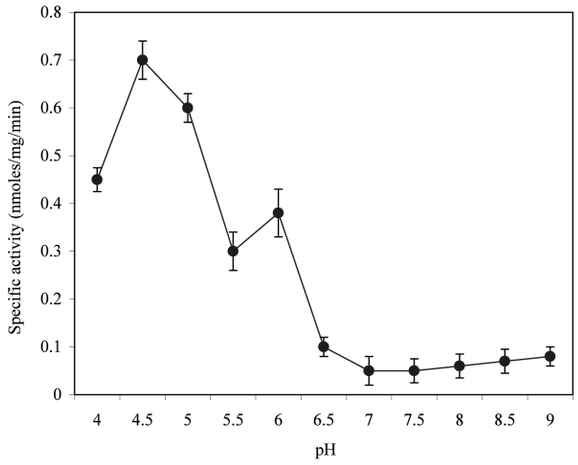

Figure 5. Effect of incubation pH on KAS II activity. The purified enzyme was incubated in various buffers; 50 mM acetate pH 4.0-5.5, potassium phosphate pH 6.0-8.5, and glycine pH 9.0. Means ± S.D. (n=3).

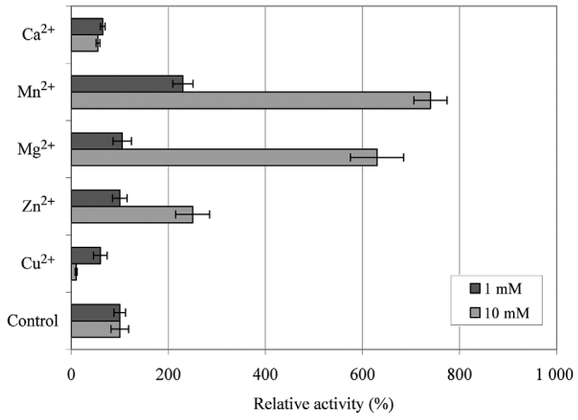

Figure 6. Effect of divalent cations on KAS II activity. The assay was carried out in the presence of divalent cations at 1 mM and 10 mM concentrations. Means ±S.D. (n=3).

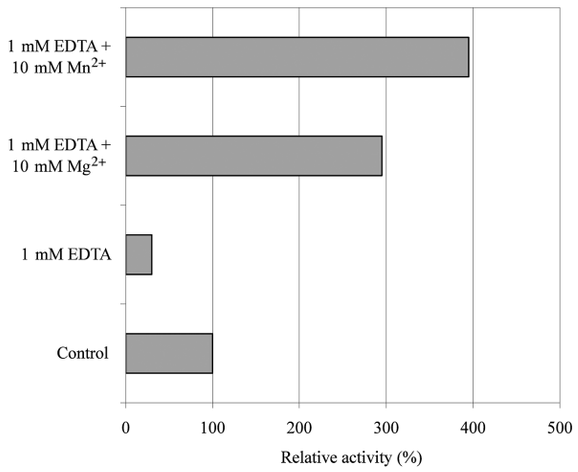

Figure 7. Effect of EDTA on KAS II activity. Panel (a) shows KAS II activity in 1 mM EDTA and panel (b) the KAS II activity regained by the addition of metal cations, Mg^{2+} and Mn^{2+}. Means ± S.D. (n=3).

content (increase in KAS II activity) is accompanied by a concomitant increase in oil content in high C18:1 rapeseed (Mollers and Schierholt, 2002).

The research evidence that KAS II plays a vital role in the regulation of palmitic acid accumulation in oil palm mesocarp suggests that low activity of KAS II impedes the conversion of palmitoyl-ACP to stearoyl-ACP, resulting in high amounts of palmitoyl-ACP which is efficiently hydrolysed to palmitic acid by a palmitoyl-ACP thioesterase (Sambanthamurthi *et al*., 1990; 2000). Such findings extend the knowledge concerning plant lipid biosynthesis in oil crops. Through the oil palm breeding programme, many individual palms have been screened for oils with high IV (Rajanaidu *et al*., 2000) to improve the unsaturation level in oil palm. In this study, KAS II activity was examined in various oil palm breeding materials which had different IV and levels of fatty acids. The IV of *E. oleifera* was approximately 80 and that of Nigerian *E. guineensis* 55. Interspecific hybrids between them possessed an iodine value of 65 (*Table 1a*). However, it was reported that oil yields of the current hybrids are too low for commercial exploitation (Soh *et al*., 2009).

The analysis showed that KAS II activity was higher in *E. oleifera* (a high oleate variety) than in *E. guineensis* (a high palmitate variety). Regression analysis showed that KAS II activity correlated negatively with C16:0 fatty acid, which is thus in agreement with the established understanding that KAS II mediates the elongation of 16:0-ACP to 18:0-ACP and has a substantial effect on the ratio of C16:C18 fatty acids. This conclusion is further supported by the correlation data that show strong positive correlations between KAS II and total C18 saturated and unsaturated fatty acids. The most immediate effect of KAS II overproduction is the accumulation of C18:0 and, in palm oil, the final low level of stearic acid (5%) is a consequence of the high activity of stearoyl-ACP desaturase which, in turn, accounts for the high oleic acid content in palm oil. Therefore, in oil palm, it is unlikely that increasing the activity of stearoyl-ACP desaturase alone will increase oleic acid levels further in oil palm mesocarp as the stearic acid content in palm oil mesocarp is less than 5%. It may however, play a positive role if KAS II activity is increased either by breeding or transgenic modification.

In addition to providing a biochemical explanation of the role of KAS II in controlling fatty acid biosynthesis in oil palm, the examination of the KAS II activity profile during fruit development was useful. The data provided information on the close relationship between KAS II activity and triacylglycerol synthesis, and also revealed the best stage for KAS II enzyme purification studies. It is clear that KAS II activity has a close relationship with triacylglycerol synthesis. However, the degree to which this factor contributes to an oil content increase and enhances oil yield performance in the field remains unknown.

Up until now, little information on the purification of KAS II enzymes from plants is available. Two decades ago, partial purification of KAS II was reported for spinach leaves (Schuz *et al*., 1982) and oilseed rape (MacKintosh *et al*., 1989). This may reflect the enzyme's low abundance as suggested by the low specific activities measured in cell-free plant extracts (Shimakata and Stumpf, 1982). Several extraction procedures were attempted to purify KAS II from oil palm mesocarp in order to obtain the best yield and activity. Purification of oil palm KAS II was carried out to achieve near homogeneity from the oil palm mesocarp and aspects of its activity were characterised.

Acetone precipitation was the most efficient method for concentrating the protein with minimum loss of activity (less than 5%). The use of mesocarp acetone powder for oil crop enzyme studies have been reported previously to stabilise activity and remove lipids (Manaf and Harwood, 2000). Therefore acetone precipitation was used in the subsequent crude extract preparation. An important requisite for successful ACP-Sepharose purification was achieved through: (i) ACP preparation from the *E. coli*, and (ii) the pre-ion exchange step by the hydroxyapatite which was necessary for protein binding. A significant loss in KAS II activity was experienced when commercial ACP was used. The purified enzyme was unstable to freezing and lost half of its original activity at -70°C after two weeks even with the addition of PMSF or benzamidine. On the basis of isoelectric focusing and SDS-polyacrylamide gels, it is suggested that oil palm KAS II had been successfully purified almost to homogeneity. The native values reported for KAS I and KAS II from rape seed (MacKintosh *et al*.,

1989) are very similar to those reported for *E. coli* (Garwin *et al*., 1980). The approximate molecular weight of *E. coli* KAS I and KAS II were 80 000 and 85 000, respectively (Garwin *et al*., 1980), whereas the spinach I and II enzymes have Mr-values of 56 000 and 57 500, respectively (Shimakata and Stumpf, 1982).

The purified enzyme was characterised with regard to its enzymatic properties. The analysis shows that KAS II from oil palm shows differences in some of these characteristics compared with KAS II from rape (Shimakata and Stumpf, 1982) and spinach (MacKintosh *et al*., 1989). KAS II activity increased in the presence of divalent cations such as Mn^{2+} and Mg^{2+}. While the results were obtained under *in vitro* conditions, it would be interesting to know how these will translate under *in vivo* conditions. Oil palm is by far the largest fertiliser-consuming crop and the role of fertilisers which contain Mg^{2+} in affecting fatty acid composition remains unknown. In summary, our data clearly demonstrate that the high level of palmitic acid in oil palm is partly due to limited KAS II activity. Therefore, KAS II is a key enzyme to consider in any future genetic modifications to alter palm oil composition. Due to the lengthy breeding cycle of the oil palm and the complexity of the genetic traits underlying fatty acid quality, both breeding and transgenic strategies are long-term ventures that may take several decades to realise. Meanwhile, high oleic transgenic oil palm is currently under development using the transgenic strategy but it is likely to take more than 10-15 years for realisation.

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