Journal of Food and Nutrition Research, 2014, Vol. 2, No. 1, 34-39
Available online at http://pubs.sciepub.com/jfnr/2/1/6

DOI:10.12691/jfnr-2-1-6

Analysis of Major Constituents in Seed Cells of *Aquilaria sinensis*

Da-Huang Chen[1], Pei-Luen Jiang[2,3], Tzyy-Rong Jinn[4,*], Jason T.C. Tzen[1,4,5,*]

[1]Graduate Institute of Biotechnology, National Chung-Hsing University, Taichung, Taiwan, R.O.C
[2]Taiwan Coral Research Center, National Museum of Marine Biology and Aquarium, Pingtung, Taiwan, R.O.C
[3]Institute of Marine Biotechnology, National Dong-Hwa University, Pingtung, Taiwan, R.O.C
[4]School of Chinese Medicine, China Medical University, Taichung, Taiwan, R.O.C
[5]Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan, R.O.C
*Corresponding author: jinn@mail.cmu.edu.tw; tctzen@dragon.nchu.edu.tw

Received October 28, 2013; Revised January 18, 2014; Accepted January 23, 2014

Abstract Agarwood, a resinous heartwood with valuable fragrance, is formed when the *Aquilaria* trees are injured. In the past two decades, many *Aquilaria* plants were cultivated for the induction of agarwood in Taiwan. Plenty of *Aquilaria* seeds are generated annually, and seem to be a reliable agricultural source. However, the constituents of these seeds have not been analyzed. Proximate composition of fresh *Aquilaria* seeds was analyzed as 44.4% moisture, 24.9% crude lipid, 16.7% carbohydrate, 10.3% crude fiber, 2.4% crude protein, and 1.3% ash. Two major subcellular organelles, abundant oil bodies and large protein bodies, were observed in electron microscopy. Protein bodies are possibly composed of soluble 2 S albumin and insoluble 11 globulin storage proteins. Oil bodies presumably encapsulate abundant storage lipids with oleosin and caleosin. The storage lipids in oil bodies were mainly neutral lipids (> 90% triacylglycerols and ~5% diacylglycerols). Fatty acids released from these neutral lipids were highly unsaturated with approximately 80% of oleic acid. Oily *Aquilaria* seed is an adequate source of neutral lipids rich in unsaturated oleic acid, and its oil bodies may serve as storage pools for the accumulation of unique agarwood lipid compounds after the tree is substantially injured for years.

Keywords: Aquilaria, *protein body, oil body, oily seed, oleic acid*

Cite This Article: Da-Huang Chen, Pei-Luen Jiang, Tzyy-Rong Jinn, and Jason T.C. Tzen, "Analysis of Major Constituents in Seed Cells of *Aquilaria sinensis*." *Journal of Food and Nutrition Research*, vol. 2, no. 1 (2014): 34-39. doi: 10.12691/jfnr-2-1-6.

1. Introduction

Agarwood is a highly valuable resinous heartwood with distinctive fragrance. The fragrance of agarwood is complex and pleasing, and almost no equivalent analogues are found in natural sources [1]. Consequently, agarwood as well as its essential oil is constantly used as incense for religious ceremonies, perfumes in the Arab world, ornamental materials, and medicinal components in oriental medicine [2]. Agarwood is not found in normal wood tissues; instead, it is formed when the trunks, branches, and roots of some *Aquilaria* trees are injured by insects, physical cuts, bacterial and fungal infections, or chemical stimulation [3]. Many unique compounds, such as sesquiterpenes and 2-(2-phenylethyl) chromones, have been identified in agarwood, but not found in the original plants [4,5].

Since the demand for agarwood far exceeded the supply in the late of the last century, the *Aquilaria* plants have declined to a threatened level according to the IUCN Red List [6]. As a consequence of the depletion of the wild resource, the price of agarwood has been tremendously elevated in the past few decades. In response to the continuous demand of agarwood, many *Aquilaria* plants were cultivated for the induction of agarwood in several countries in the past few decades. Of course, many protocols of fungal infections were commercially developed in secret and aimed to transform the original plants into valuable agarwood.

Seeds are the part of a flowering plant that typically stores the initial source of nutrition for germination and subsequent seedling growth [7]. The stored nutrition is occasionally preserved in the form of proteins, yet much more commonly in the form of carbohydrates or lipids [8]. Seed cells deposit storage resources of carbohydrates, proteins and neutral lipids in distinct subcellular particles termed starch granules, protein bodies and oil bodies, respectively [9,10]. Degradation of carbohydrates and neutral lipids provides energy as well as carbon source while that of proteins provides amino acid source for the *de novo* biosynthesis of seedling proteins after germination.

A protein body contains a matrix of storage proteins surrounded by a lipid bilayer. Storage proteins found in protein bodies of diverse seeds have been classified into four groups, water-soluble albumins, dilute saline-soluble globulins, alcohol-soluble prolamins, and dilute acid- or alkali-soluble glutelins, based on their solubility in various

extraction solvents [11]. The globulins are further divided into two subgroups according to their sedimentation coefficients: 7 S vicilin-type and 11 S legumin-type globulins. Most seeds of dicotyledonous species comprise three classes of storage proteins, 11 S globulin, 7 S globulin, and 2 S albumin, and isoforms are present in each of the three classes [12].

An oil body is 0.5 to 2.5 μm in diameter and contains a lipid matrix surrounded by a monolayer of phospholipids embedded with some unique proteins [10]. Oil bodies are remarkably stable both in vivo and in vitro as compressed oil bodies in cells of a mature seed or in the milky layer during isolation never coalesce or aggregate. The remarkable stability of oil bodies in aqueous environments is a consequence of the presence of unique proteins on the their surface [8]. To date, three classes of integral proteins, termed oleosin, caleosin and steroleosin, have been identified in oil bodies of angiosperm seeds [7].

In the past two decades, many *Aquilaria* plants were cultivated in different areas of Taiwan. Plenty of *Aquilaria* seeds are generated annually, and seem to be a reliable agricultural source. However, the constituents of these seeds have not been analyzed. In this study, we attempted to examine the structural organization of *Aquilaria* seeds at a subcellular level and evaluated the nutrient value of these seeds by analyzing their major constituents.

2. Methods

2.1. Plant Materials

Seeds of *Aquilaria sinensis* were provided by a local grower, Mr. Cheng-Shen Lin (Wufen, Taichung). Sesame (*Sesamum indicum* L., Tainan1) was a gift from Dr. Tien-Joung Yiu of the Crop Improvement Department, Tainan District Agricultural Improvement Station.

2.2. Proximate Analysis

Moisture, crude fat, ash, crude protein, crude fiber, and ash were determined according to the AOAC methods [13].

2.3. The Transmission Electron Microscopy

Seeds of *Aquilaria sinensis* were collected and fixed in 25% glutaraldehyde and 16% paraformaldehyde in 100 mM sodium phosphate containing and 5% sucrose (pH 7.3) for 3 h at 4°C. They were then rinsed with 100 mM sodium phosphate buffer at 4°C. Seeds were then post-fixed in 1% OsO_4 in 50 mM sodium phosphate (pH 7.3) for 1 h at 4°C. The seed aliquots were then washed three times for 15 min each with the same buffer and dehydrated by a graded ethanol series (70, 80, 90, 95 and 100%) before embedding in LR white Resin (London Resin Co.). Thin sections (70 nm) cut by a Leica Reichert Ultracut R were collected on nickel grids, post-stained with 2.5% uranyl acetate and 0.4% lead citrate, rinsed 3 times with water, and the samples were viewed on a JEM-1400 transmission electron microscope (JEOL, Japan).

2.4. Subcellular Fractionation of Seed Proteins

Mature *Aquilaria* seeds were extracted with a medium containing 0.6 M sucrose and 10 mM sodium phosphate buffer (pH 7.5). The extract was separated into three fractions (supernatant, pellet and oil bodies) by centrifugation at 10,000 × g for 15 min [14]. The supernatant, pellet and oil bodies were separately collected for further analyses.

2.5. Purification of Seed Oil Bodies

Crude extract of oil bodies from *Aquilaria* seeds as well as those from sesame seeds were subjected to further purification using a protocol described previously [14]. The method included two-layer flotation by centrifugation, detergent washing, ionic elution, treatment with a chaotropic agent, and integrity testing with hexane.

2.6. Analysis of Seed Proteins in SDS-PAGE

For SDS-PAGE analysis, the supernatant, pellet and oil bodies were extracted with the sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol with or without β-mercaptoethanol according to the Bio-Rad instruction manual [15]. The separating gel was composed of 12.5% polyacrylamide, and the electrophoresis was performed under 200 V for 120 min. For the analysis of the 10 kDa band of supernatant, a separating gel of 18% polyacrylamide was used, and the electrophoresis was performed under 200 V for 70 min. Following electrophoresis, the gels were stained with Coomassie Blue R-250.

2.7. Mass Spectrometric Analysis

After resolved by SDS-PAGE, the candidate oleosin (17 kDa) and caleosin (27 kDa) in oil bodies of *Aquilaria* seeds were manually excised from the gel and ground into pieces. Followed by in-gel digestion by trypsin, the resulting fragments were subjected to mass spectrometric analysis for protein identification by using the same protocols as described previously [8].

2.8. Analysis of Neutral Lipids

Oil bodies extracted from *Aquilaria* seeds were subjected to the analysis of neutral lipids by thin layer chromatography (TLC). Purified oil bodies of 50 μl were extracted with 150 μl of chloroform/methanol (2/1, v/v). After centrifugation, the lower chloroform fraction was collected and spotted onto a TLC plate coated with silica gel. The TLC plate was developed in a solvent system containing hexane: diethyl ether: acetic acid (80/20/2, v/v/v) [14]. After development and drying, lipids were visualized by reacting with iodine.

2.9. Analysis of Fatty Acid

Neutral lipids of *Aquilaria* oil bodies (4 ml) were subjected to analysis of fatty acid composition by gas liquid chromatography. According to the AOCS official method Ce-1b-89, the methylation of fatty acids was carried out with boron trifluoride-methanol reagent [16]. After extraction with octane, the fatty acid methyl esters were separated by GLC (HP 6890, Hewlett Packard, CA, USA) using a 30 m × 0.25 mm × 0.25 μm capillary silica column (Supelco wax 10; Supelco, Bellefonte, PA, USA),

and identified by comparison of their retention times with authentic standards.

3. Results

3.1. Proximate Composition and Subcellular Organization of *Aquilaria* Seeds

Proximate composition of fresh *Aquilaria* seeds was analyzed and shown in Table 1. Having its lipid content higher than 40% in the dry weight, *Aquilaria* seeds are suitably regarded as a kind of oily tissue. The majority of subcellular components in *Aquilaria* seeds were examined in electron microscopy. The examination showed that seed cells were predominantly filled with two types of subcellular organelles, abundant oil bodies (gray spherical particles of mostly 1-3 μm in diameter) and large protein bodies (dark black particles of 3-6 μm in diameter) (Figure 1). No apparent starch granules were observed within the seed cells. The presence of abundant oil bodies in *Aquilaria* seeds is in accord with the proximate composition analyzed.

Table 1. Proximate composition of *Aquilaria* seeds (%)

Component	% of total
Moisture	44.4
Crude fat	24.9
Carbohydrate	16.7
Crude fiber	10.3
Crude protein	2.4
Ash	1.3

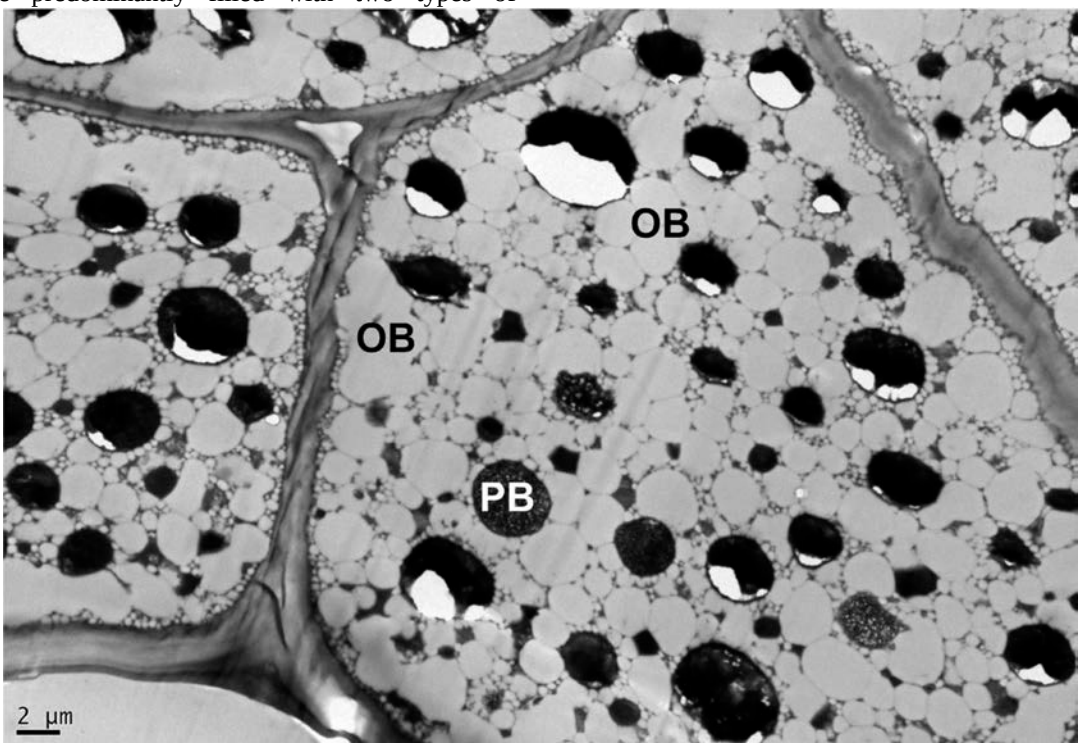

Figure 1. Electron microscopy of seed cells of *Aquilaria sinensis*. Protein bodies and oil bodies in a seed cell of *Aquilaria sinensis* were labeled as PB and OB, respectively. Bar at the bottom represents 2 μm

3.2. Major Proteins of *Aquilaria* Seeds

Total proteins of *Aquilaria* seed were fractionated into three fractions (supernatant, pellet and oil layer), and then subjected to SDS-PAGE analysis in the absence of β-mercaptoethanol. The major protein bands in the supernatant, pellet and oil layer were approximately 10, 55 and 17 kDa, respectively (Figure 2). To verify if the major proteins in the supernatant and pellet fractions were 2 S albumin and 11 S globulin storage proteins, the 10 kDa band (supernatant) and the 55 kDa band (pellet) were separately eluted from gels, and then subjected to SDS-PAGE analysis in the presence and absence of β-mercaptoethanol. The 55 kDa band split into two polypeptide groups of approximately 35 kDa and 20 kDa, and the 10 kDa band also split into shorter polypeptides in the presence of β-mercaptoethanol (Figure 3). The results suggest that the 55 kDa band from the pellet and the 10 kDa band from the supernatant are presumably well-known seed storage proteins, 11 S globulin and 2 S albumin, comprising two polypeptide subunits linked by disulfide bonds.

Enriched proteins extracted from oil bodies of *Aquilaria* seeds as well as those extracted from sesame oil bodies were resolved in SDS-PAGE (Figure 4 A). Similar protein patterns were observed in these two oil bodies including putative oleosin isoforms (15 and 17 kDa) and caleosin (27 kDa). To further verify the presence of oleosin and caleosin in the oil bodies of *Aquilaria* seeds, the candidate proteins of 17 kDa and 27 kDa were subjected to mass spectrometric analysis after trypsin digestion. In-gel digestion of the 17 kDa candidate protein produced a fragment, QPPGADQLDQAR, which matched a tryptic fragment of the theoretical oleosin found in *Prunus dulcis* (accession No. Q 43804) (Figure 4 B). Similarly, in-gel digestion of the 27 kDa candidate protein produced a fragment, CFDGSLFEYCAK, which matched a tryptic fragment of the theoretical caleosin found in *Arabidopsis thaliana* (accession No. NP 194404) (Figure 4 C). The results suggest that oleosin and caleosin are present in oil bodies of *Aquilaria* seeds.

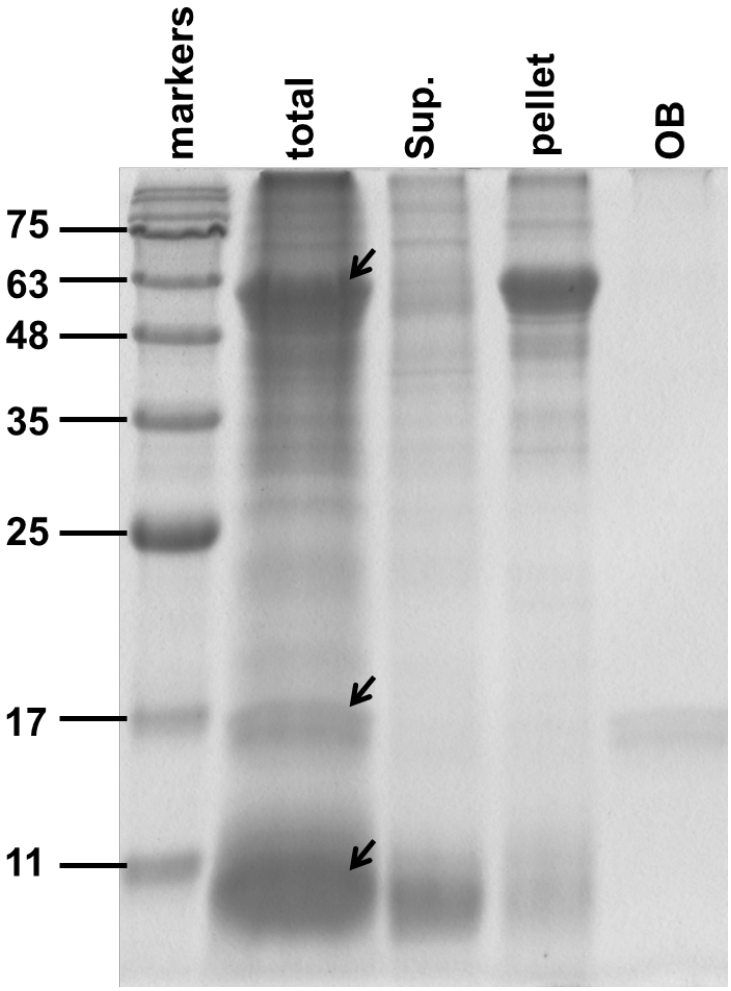

Figure 2. SDS-PAGE of *Aquilaria* seed proteins fractionated by centrifugation. Total extract of *Aquilaria* seed proteins was centrifugated at 10,000 × *g* to yield three fractions, supernatant (Sup.), pellet and oil bodies (OB). The major protein bands in the three fractions were also indicated by arrows in the total extract. Labels in the left indicate the molecular masses of marker proteins

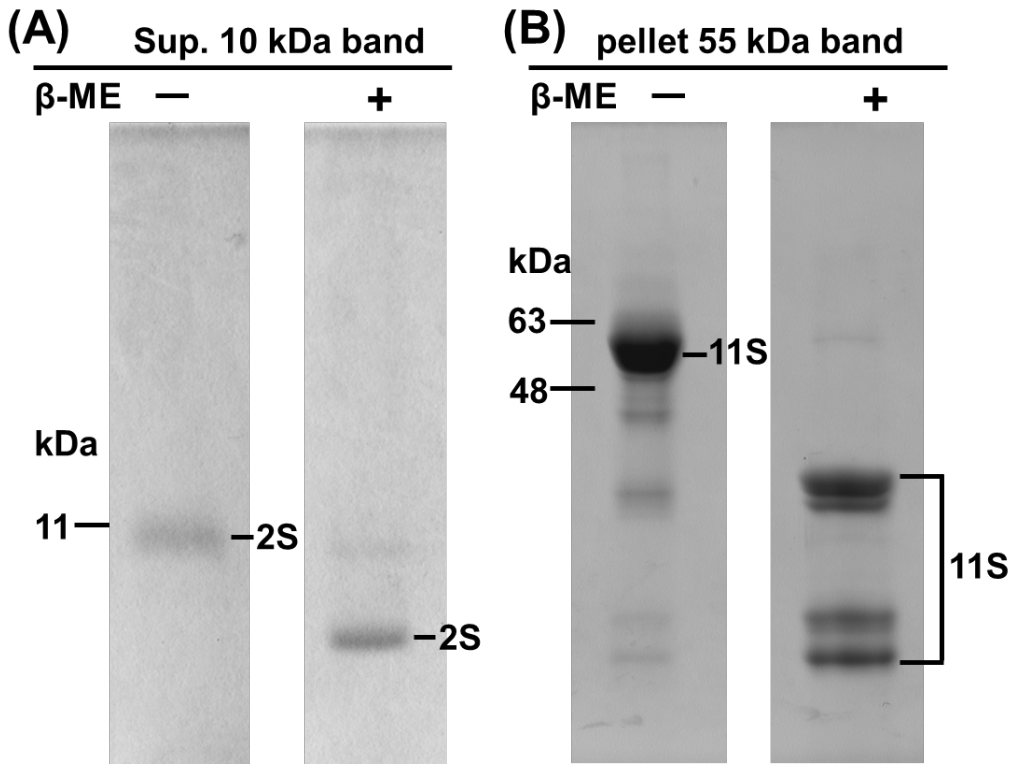

Figure 3. SDS-PAGE of the major soluble and insoluble proteins of *Aquilaria* seeds. The major band of the supernatant (10 kDa) and tthat of the pellet (55 kDa) were subjected to SDS-PAGE analysis in the absence and presence of β-mercaptoethanol (β-ME). Putative polypeptides of 2 S albumin and 11 S globulin are indicated

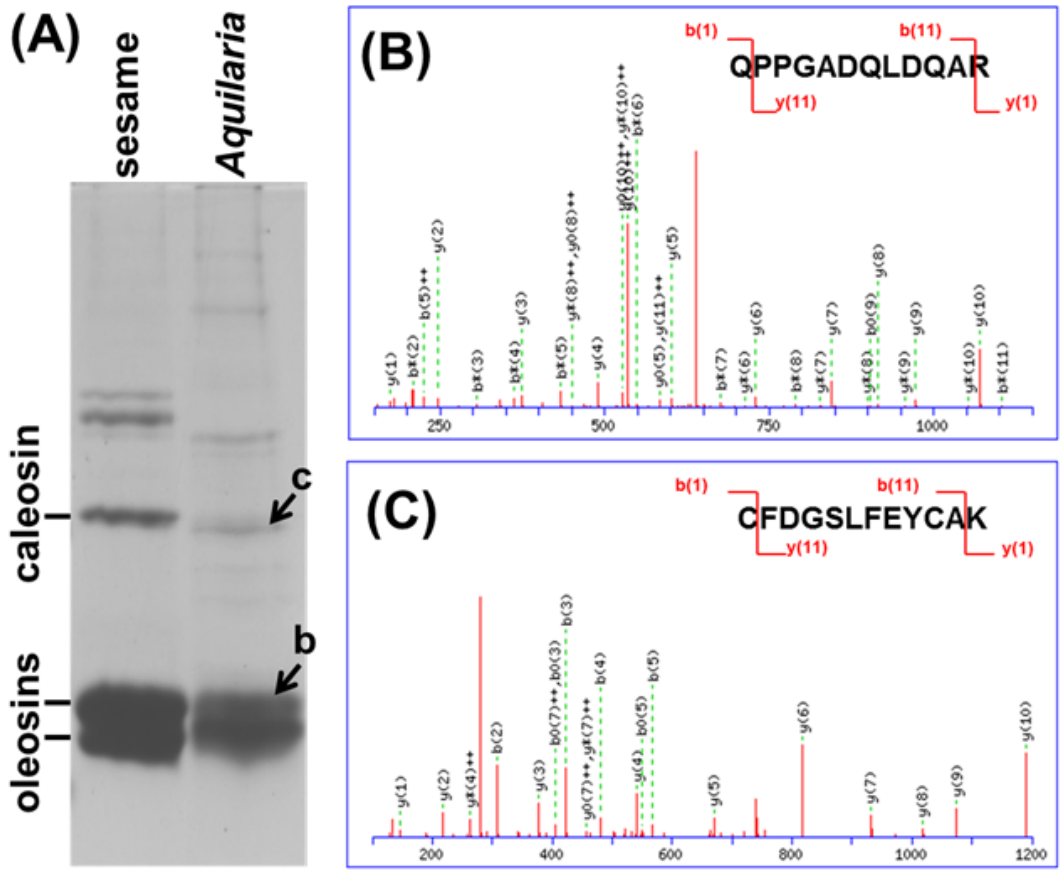

Figure 4. SDS-PAGE of proteins from sesame and *Aquilaria* oil bodies (A). Proteins extracted from oil bodies of sesame and *Aquilaria* seeds were resolved by SDS-PAGE. Putative oleosin (17 kDa) and caleosin (27 kDa) indicated by arrows (labeled with b and c) were subjected to mass spectrometric analysis after trypsin digestion (B and C). The peptide fragment identified to be related to oleosin or caleosin was shown on the right top corner. All matched b and y ions were labeled in the figures

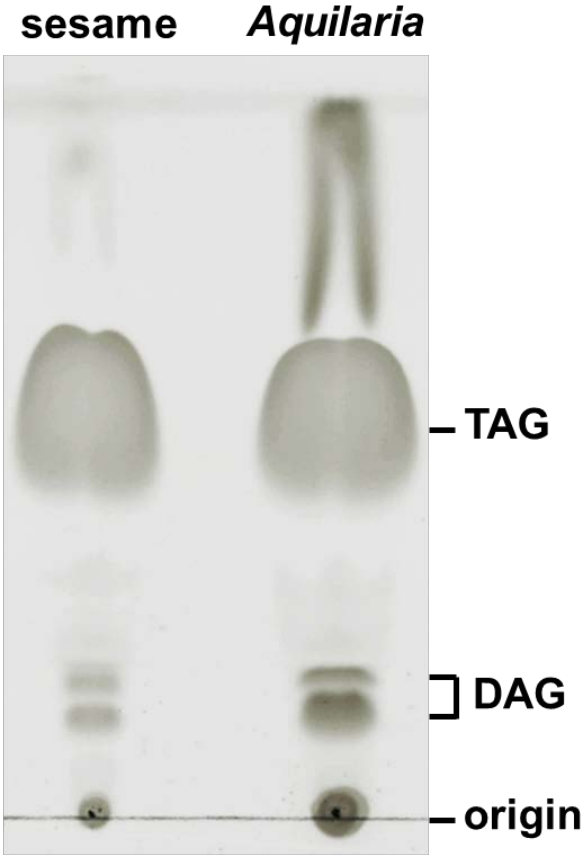

Figure 5. Neutral lipids extracted from oil bodies of sesame and *Aquilaria* seeds analyzed by TLC. The positions of triacylglycerols (TAG) and diacylglycerols (DAG) are indicated in the right margin

3.3. Lipids of *Aquilaria* Seed Oil Bodies

Thin-layer chromatography showed that the milky oil bodies purified from the *Aquilaria* seeds were mainly composed of neutral lipids, > 90% triacylglycerols and ~5% diacylglycerols (Figure 5), in a manner similar to the neutral lipids extracted from sesame oil bodies [17]. Fatty acids of the neutral lipids extracted from oil bodies of *Aquilaria* seeds were highly unsaturated with approximately 80% of oleic acid (Table 2).

Table 2. Fatty acid composition of lipid extracted from oil bodies of *Aquilaria* seeds

Fatty acid type	Retention time (min)	% of total
C 14 (myristic Acid)	27.819	0.109
C 16 (palmitic acid)	32.196	9.061
C 18: 2 (linoleic acid)	35.231	1.405
C 18: 1 (oleic acid)	35.637	80.311
C 18 (stearic acid)	36.088	5.285

4. Discussion

According to the analysis of proximate composition, observation of subcellular organization, and identification of major constituents in this study, *Aquilaria* seeds are regarded as a kind of oily tissue, and may serve as an adequate source of neutral lipids rich in unsaturated oleic acid. Oleic acid is a common monounsaturated fatty acid in human diet, and its sodium salt (soap) is daily used as an emulsifying agent. Higher intake of oleic acid seems to be associated with a decreased risk of coronary heart

disease caused by high cholesterol level in blood [18]. Obviously, seed oils of *Aquilaria sinensis* is beneficial for human health.

Similar to many oily dicotyledonous seeds, such as sesame, *Aquilaria* seeds are composed of two major types of storage proteins, 11 S globulin and 2 S albumin, presumably accumulated in protein bodies [18]. Relatively insoluble 11 S globulin represents the most abundant protein and 2 S albumin stands for the major soluble protein in *Aquilaria* seeds. Some space within protein bodies of *Aquilaria* seeds seems to be empty and is not stained with osmium in electron microscopy (Figure 1). Since protein bodies fully filled with storage proteins should be osmium-stained as solid black entities, it is likely that the *Aquilaria* seeds examined in this study are not utterly mature, and thus their protein bodies are not fully filled with storage proteins. Therefore, the protein content of *Aquilaria* seeds is probably higher than that analyzed in the current study (Table 1) when they are completely mature.

Regardless the potential utilization of *Aquilaria* seeds, agarwood is still the most valuable product of *Aquilaria* plants. The value of agarwood is basically resulted from the accumulation of unique injury-induced lipid compounds, presumably acting as defensive agents. In this study, oil bodies are found as the majority of subcellular organelles in *Aquilaria* seeds, and thus may serve jointly as a massive pool for the accumulation of lipid compounds other than triacylglycerols. It is reasonable to speculate that the unique agarwood lipid compounds (defensive agents) may be also transported to seed cells and deposited into the subcellular oil bodies for the protection of offspring after a long term infection or injury. It remains to be seen if the valuable agarwood lipid compounds can be harvested from seed oil bodies of manipulated *Aquilaria* plants substantially injured for years.

Acknowledgement

The work was supported by a grant from the National Science Council, Taiwan, ROC (NSC 100-2313-B-005-015-MY3 to JTC Tzen).

References

[1] Chen, H.Q., Wei, J.H., Yang, J.S., Zhang, Z., Yang, Y., Gao, Z.H., Sui, C. and Gong, B. "Chemical Constituents of Agarwood Originating from the Endemic Genus Aquilaria Plants," *Chemistry and Biodiversity*, 9 (2). 236-250. Feb. 2012.

[2] Okudera, Y. and Ito, M. "Production of agarwood fragrant constituents in Aquilaria calli," *Plant Biotechnology*, 26 (3). 307-315. 2009.

[3] Ito, M. "Studies on perilla, agarwood, and cinnamon through a combination of fieldwork and laboratory work," *Journal of Natural Medicines*, 62 (4). 387-395. Oct. 2008.

[4] Tamuli, P., Boruah, P., Nath, S.C. and Leclercq, P. "Essential oil of eaglewood tree: a product of pathogenesis," *Journal of Essential Oil Research*, 17 (6), 601-604. Nov. 2005.

[5] Dai, H.F., Liu, J., Han, Z., Zeng, Y.B., Wang, H. and Mei, W.L. "Two new 2-(2-phenylethyl) chromones from Chinese eaglewood," *Journal of Asian Natural Products Research*, 12 (2). 134-137. Feb. 2010.

[6] IUCN, The 2000 IUCN Red List of Threatened Species, Gland, Switzerland, 2000.

[7] Hsiao, E.S.L. and Tzen, J.T.C. "Ubiquitination of oleosin-H and caleosin in sesame oil bodies after seed germination," *Plant Physiology and Biochemistry*, 49 (1). 77-81. Jan. 2011.

[8] Lin, I.P., Jiang, P.L., Chen, C.S. and Tzen, J.TC. "A unique caleosin serving as the major integral protein in oil bodies isolated from Chlorella sp. cells cultured with limited nitrogen," *Plant Physiology and Biochemistry*, 61, 80-87. Dec. 2012.

[9] Chen, D.H., Chyan, C.L., Jiang, P.L., Chen, C.S. and Tzen J.T.C. "The same oleosin isoforms are present in oil bodies of rice embryo and aleurone layer while caleosin exists only in those of the embryo," *Plant Physiology and Biochemistry*, 60. 18-24. Nov. 2012.

[10] Chua, C.A.N., Jiang, P.L., Shi, L.S., Chou, W.M. and Tzen, J.T.C. "Characterization of oil bodies in jelly fig achenes," *Plant Physiology and Biochemistry* 46 (5-6). 525-532. May-Jun. 2008.

[11] Tai, S.S.K., Wu, L.S.H., Chen, E.C.F. and Tzen, J.T.C. "Molecular cloning of 11 S globulin and 2 S albumin, the two major seed storage proteins in sesame," *Journal of Agricultural and Food Chemistry*, 47 (12), 4932-4938. Dec. 1999.

[12] Tzen, J.T.C., Wang, M.M.C., Tai, S.S.K., Lee, T.T.T. and Peng, C.C. "The abundant proteins in sesame seed: storage proteins in protein bodies and oleosins in oil bodies," *Advances in plant physiology*, 6. 93-105. 2003.

[13] Association of Official Analytical Chemists (AOAC). Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed.; AOAC: Washington, DC, 1990.

[14] Tzen, J.T.C., Peng, C.C., Cheng, D.J., Chen, E.C.F. and Chiu, J.M.H. "A new method for seed oil body purification and examination of oil body integrity following germination," *The Journal of Biochemistry*, 121 (4). 762-768. Apr. 1997.

[15] Hsiao, E.S.L., Lin, L.J., Li, F.Y., Wang, M.M.C., Liao, M.Y. and Tzen, J.T.C. "Gene families encoding isoforms of two major sesame seed storage proteins, 11 S globulin and 2 S albumin," *Journal of Agricultural and Food Chemistry*, 54 (25). 9544-9550. Dec. 2006.

[16] AOCS official method Ce-1b-89, Fatty acid composition by GLC: Marine oils, Champaign IL, 1992.

[17] Peng, C.C. and Tzen J.T.C. "Analyses of the three essential constituents of oil bodies in developing sesame seeds," *Plant and Cell Physiology*, 39 (1). 35-42. Jan. 1998.

[18] Terés, S., Barceló-Coblijn G., Benet, M., Alvarez R, Bressani R, Halver, J.E. and Escribá, P.V. "Oleic acid content is responsible for the reduction in blood pressure induced by olive oil." *Proceedings of the National Academy of Sciences*, 105 (37). 13811-13816. Sep. 2008.