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Isolation of oleosin and determination of oil body size in *Brassica juncea*

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Abstract

Oil bodies are structural entities that store triacylglycerols (TAG) in oilseeds. Each oil body contains a matrix of triacylglycerol (TAG) surrounded by a layer of phospholipids and its associated oil body proteins. Description of oil body and its associated proteins has not been well documented in *Brassica juncea* unlike *Arabidopsis* which is a related species. In this study extraction of oil bodies (OB) was done by flotation method following which the presence and integrity of oil bodies was confirmed using compound light microscope. The size of OB ranged from 0.58 (Maya) to 1.05 µm (HB9902). The major phospholipid as profiled by TLC was phospotidylcholine. Purification of structural proteins was done by altering the time of urea (8M) treatment. The proteins isolated from the oil bodies showed multiple bands in SDS-PAGE that included oleosin protein. Downstream purification of oil body proteins was standardized to remove further unspecific bands by additional urea treatment with time of incubation 30 min, 1h and overnight at room temperature.Out of the three, overnight incubation showed less appearance of unspecific bands. A comparative study among selected genotypes was also done showing oil content to be inversely related to oil body size and positively correlated to oleosin content. Hence by manipulating the oleosin proteins, it may be possible to increase the oil content.

Keywords: *Brassica juncea, oil bodies, oleosin*

Introduction

Oleaginous plants like oilseed brassica contain storage lipid vesicles called oil bodies (also called as oleosomes) that are composed of triacylglycerols surrounded by a mono layer of phospholipids embedded with surface active proteins (e.g. oleosins) (Tzen and Huang, 1992; Tzen *et al*., 1993). Oil bodies (OB) have been isolated from wide number of plant seeds like maize (Nikiforidis and Kiosseoglou, 2009), Soybean (Chen et al. 2012), pumpkin (Adam *et al*., 2012), rapeseed (Tzen *et al*., 1990, Joliv *et al*., 2006, Hu *et al*., 2009), *Arabidopsis* (Miquel *et al*., 2014) and rice (Nantiyakul *et al.,* 2012). The size of oil bodies differs from species to species depending upon the set of surrounding proteins (oleosin) and the nature of lipids stored. By and large all oil bodies play the same role that is reservoir of energy (Murphy, 2012). There has been difference of opinion regarding the size of oil bodies.It was reported in *Brassica napus* that cultivars having larger oil bodies have low oil content usually and while some cultivars did not show this correlation. However, the variation was suggestedto be due to oleosin content (Hu *et al*., 2009) and it is documented in previous reports that oleosin maintains the stability of OB, thus preventing the organelles from coalescing by providing stearichindrance during desiccation (Tzen and Huang 1992, Tzen *et al*., 1993, Ross *et al*., 1993, Ting *et al*., 1996). Studies in *Arabidopsis*, showed mutation in gene encoding oleosin protein resulted in unusually large size of OB (Siloto *et al*., 2006). Further, disruption of the gene encoding oleosin also leads to altered accumulation of lipids and proteins which in turn causes delay in seed germination (Siloto *et al*., 2008). Supporting to this theory is the work by Shimada *et al*. (2008) in Arabidopsis where they reported an inverse relationship between OB size and total oleosin levels. Though, there are reports in *B. napus* studying the size of OB exhibiting wide range of oil content in mature seeds, not much work in *B.juncea* has been done due to which the oil content-oil body size relationship not well established.

With improved lifestyle the consumption of vegetable oils has increased. Although oilseed brassica is the third highest consumed oilseed in the world itonly accounts to 13% of the vegetable oil consumed (Hajduch *et al*., 2006). More than, 80% of Indian villages using mustard oil at a monthly consumption rate of three to four kilograms per family (Damodaran and Hedge, 2005) in the country. The constant rise in human population poses the challenge of meeting the demand for vegetable oil.

Hence increasing oil content is an important breeding goal in oilseed Brassica. Keeping this in mind our objective was to standardize a method for the purification of oleosin protein and measurement of OB size using compound light microscope in *B. juncea.*

Materials and Methods

Plant material

Seeds of *B. juncea* varieties NATP-124, Pusa jai kisan and RL-1359, Maya, IC21023 and HB9902 were used for the study. For standardization of the protocol variety Pusa Jai Kisan was used.

Extraction of Oil Bodies

Isolation of oil bodies was done by flotation method as per the protocol of Tzen *et al.* (1990). 2 g mature seed was homogenised in 15ml cold (4ºC)grinding medium (1) [1mM EDTA, 10mMKCl,1 mM $MgCl_2$2mM DTT, 0.6 M sucrose, 0.15M tricine- KOH, pH 7.5] using mortar and pestle. Crude homogenate was filtered through 2layers of Muslin cloth and added to 15ml cold (4ºC) flotation medium(1)[same as grinding medium 1 with additional 0.4 M sucrose] followed by Centrifugation at10,000g for 30min. The top oleaginous layer was carefully removed using spatula. The oleaginous layer was resuspended on 15ml grinding medium (2) [same as grinding medium 1 with additional 2M NaCl] using glass homogenizer. The suspension was added to 15ml of flotation medium (2) [same as floating medium 1 with additional 2M NaCl] followed by centrifugation at 10,000g for 30min. Top layer was taken with the help of a sterilized spatula and resuspended in 15ml grinding medium(1) using a glass homogenizer. The suspension was then added to 15ml flotation medium (1) and gentrifuged at 10,000g for 30min. The above steps were repeated. Oil bodies were resuspended at room temperature (RT) in grinding medium (1) with 8MUrea, shaken for 30min. Suspension was centrifuged at 10,000g for 30min. Oil body layers were resuspended with glass homogenizer in 15ml cold (4 ºC) grinding medium(1). Suspension was added to 15ml cold (4 ºC) Floating medium (1) followed by centrifugation @10,000g for 30min. The procedure was repeated and final oil body layers were suspended in 3ml grinding medium (1). Final oil body preparation was used for oleosin isolation.

Measurement of OB size using compound light Microscope

For visualizing OB in light microscope (Olympus model no. BX51) oil body extract was diluted with double distilled water (1:50). For size measurement ocular scale was calibrated using stage micrometer. Microscope fields were selected in which illumination was uniform and oil bodies were not crowded.Twenty-five oil bodies per sample were measured, each in its best spherical dimension as it was oriented on the slide. The prepared glass slides were placed under compound microscope and visualized under 40 x magnifications to measure oil bodies. The observed data was multiplied by a factor 0.25 and the mean of all observations was taken as final reading.

In 40 x observation
20 ocular = 5 stage micrometer

1 ocular = (20/5) =0.25 stage micrometer

Extraction of Oil body proteins

The method of Tzen *et al*. (1990) was slightly modified for extraction and purification of oil body proteins (Table 1, Fig 1). Before the extraction of proteins as per the protocol of Tzen and Huang (1992) an additional step was included in which500 µl of OB extract was treated

Table 1. Showing the various purification steps with 8M urea treatment

Sample	Treatments	volume of OB extract	Time of incubation	Additional purification steps	Additional incubation	Multiple bands (+ means presence and - means absent	oleosin content (%)
Pusa Jai Kisan	8M urea (*original method)	10	30 min	-	-	+	2.9
	8M Urea	10	30min	1:1 (urea:OB extracts)	30 min	+	1.18
	8M Urea	10	30min	1:1 (urea:OB extracts)	1h	+	0.72
	8M Urea	10	30min	1:1 (urea:OB extracts)	overnight	-	0.57

*Tzen *et al*., 1990, Tzen and Huang, 1992

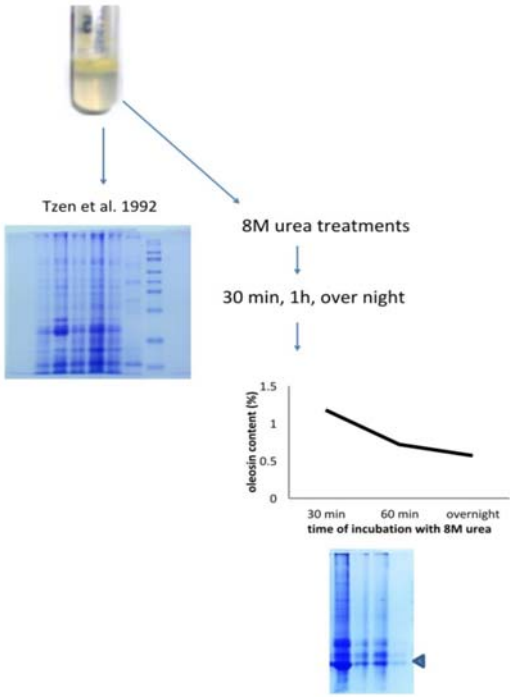

Fig.1. Overview of the extraction process and purification of oleosin proteins.

with 500 µl of 8M urea followed by different incubation times of 30min, 1h and over night at room temperature and by washingwith water several times. Rest of the extraction process was same as the protocol of Tzen and Huang (1992). A 500 µl of dimethyl ether was added to the extract in 1.5 ml vials. It was vortexed, followed by centrifugation at13, 000g for 5mins. The upper petroleum ether (diethyl ether) layer containing neutral lipid was removed. This was repeated for 2 more times. The petroleum ether (diethyl ether) fractions (~3ml) were pooled and dried. To the interfacial layer and aqueous phase 0.75ml chloroform/methanol (2:1v/v) was added and vortexed. The lower chloroform phases containing phospholipids were washed 3times with 1ml methanol/ water (1:1v/v), pooled and left for drying.The protein rich interfacial layer was resuspended in 0.25ml water. 0.75ml chloroform/methanol (2:1v/v) was added and vortexed followed by centrifugation at 13,000g for 5mins.This step was repeated two more times. After washing, oil body protein pellet was resuspended in 0.5ml water. The suspension was vigorously vortexed for 5min and precipitate in 4 vol. of cold acetone (100%) and kept for 16 h at-20°C. After precipitation in acetone, samples were centrifuged at 8500g for 15min. Total oleosin content was quantified by standard method (Lowry *et al*., 1951). Bovine serum albumin was used as standard. Soluble protein was resuspended in laemmililysis buffer and denatured at 95ºC for 4 min.

Confirmation of Oil body phospholipids by Thin Layer chromatography

The lower chloroform phase containing phospholipids were separated by Thin Layer Chromatography (TLC) (Silica G60). The plate was activated by first soaking in ammonium sulfate for 30min, followed by heating at 120ºC for 3h. Phospholipid standards namely Lá-Phosphatidylcholine (PC), Lá-Phosphatidyserine (PS), Lá-Phosphatidic acid (PA) were diluted in chloroform to get a concentration of 1mg/ml and 10µl each was loaded on the plate. For samples, 10µl of 20mg/ml concentration was loaded onto the plate. The mobile phase of acetone/ toluene/water (91:30:8) was used in the separation of the phospholipids from their origin. Bands were visualized in presence of iodine fumes.

Validationof oleosin proteins using SDS-PAGE

SDS-PAGE of oleosin protein was carried out in a 12% polyacrylamide gel in a discontinuous buffer system according to the method of Laemmli (1970). A 20 µl (each of equal concentration 20µg/ µl) was loaded in each well of the stacking gel stacked between1.5mm glass plates. Electrophoresis was run at 80V until the dye front reached the bottom of the gel. Wide range prestained protein ladder (GCC Biotech) was run on the side as reference. The gel was stained with 2% Coomassie Brilliant Blue solution, followed by destaining after 1h with mixture of methanol, acetic and water in the ratio of 5:1:4 (v/v).

Oil content

Oil content in seeds was determined by non-destructive method using FT-NIR (Bruker, Matrix-1) calibrated in our lab according to the method of Singh *et al*. (2014).

Results and Discussion

Size of oil bodies

The confirmation of OB integrity was observed under compound light microscope (Fig 2). OB sizemeasurement was standardized using the variety Pusa Jai Kisan. After the standardization, other genotypes NATP-124, RL-1359, Maya, IC21023 and HB9902 were taken for confirmation. The size of oil body ranged from 0.58 µm (Maya) to 1.05µm (HB9902) (Table 2). Our observations are in agreement with previous reports in rapeseed (0.65 µm), mustard (0.73 µm) (Tzen *et al*., 1993); Explusrape (1.3 µm), cabriotel rape (0.8 µm) (Jolivet *et al*., 2006) and in developing *B. napus* 0.23 to 3 µm (Katavic *et al*., 2006). A comparison of observations with other reports confirmedthe accuracy of OB measurement. Deviations from earlier reports may be due to the variation in oleosin content.

Table 2. Showing the variation of oil body size in selected genotypes of *B.juncea*

Genotypes	Oil content (%)	Oleosin content (%)	OB size (µm)
NATP 124	38.11	0.36	0.73
Pusa Jai Kisan	38.48	0.57	0.89
RL-1359	38.59	0.68	0.90
Maya	39.3	0.45	0.58
IC212031	39.92	0.33	0.69
HB9902	37.59	0.21	1.05

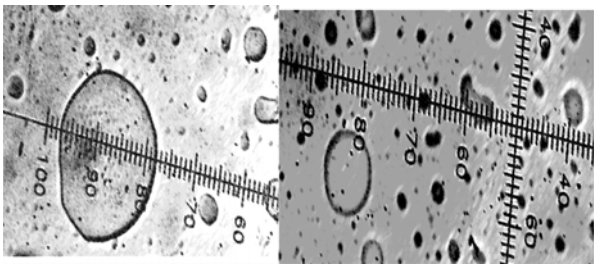

Fig. 2. Showing the integrity of OB as observed under light microscope (Olympus model # BX51) at 40X. Each small division was counted and multiplied by a factor 0.25(explained in materials and method).

Separation of Phospholipids from isolated Oil bodies

Presence of phospholipids like PC, PA, PE (phohatidylethanolamine) has been reported in cultivars of *B. napus* (Katavic *et al*., 2006). Though we did not have the standard PE with us during the analysis, in this experiment none of the samples showed any band between PC and PA as observed by Katavic *et al*. (2006). However all the samples showed prominent band indicating PC and PS (Fig 3). The presence of PC was very prominent with a thick band in all samples as in many previous reports on rapeseed oilbody phospholipids (Tzen *et al*., 1993, Katavic *et al*., 2006). Katavic *et al*. (2006) did not observe the presence of PS, but in this experiment it showed the presence of PS, though very light band which was in confirmation to the report of Tzen *et al*. (1993) in rapeseed (Fig. 3). This result shows that PC is an integral part of the OB lipid membrane.

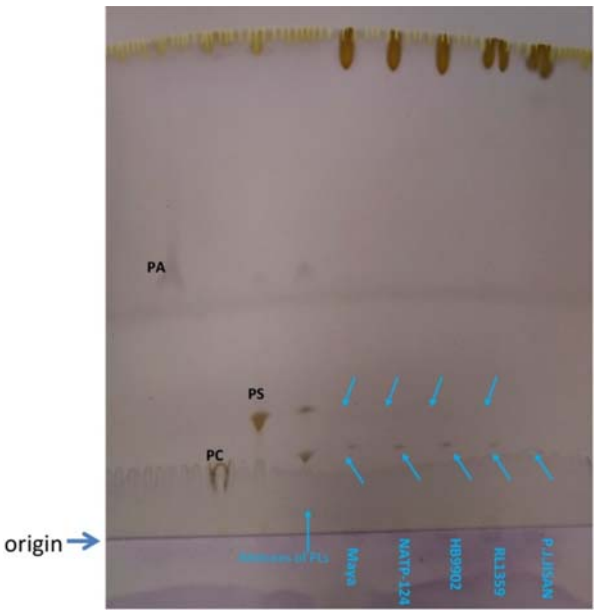

Fig. 3. TLC of PLs fractions from Oilbodies isolated fromdifferent *B.juncea*genotypes. Lá-Phosphatidyl-choline (PC), Lá-Phosphatidyserine (PS), Lá-Phosphatidic acid (PA)

Extraction, purification and quantification of Oleosin

The purification of oil body to remove exogenous proteins have previously been reported using urea at various concentrations; 7M (Jolivet *et al*., 2006), 8 M urea (Katavic *et al*., 2006), 9 M urea (Millichip *et al*., 1996; De Chirico *et al*., 2018). All these methods have used incubation time from 10 to 30 min (Jolivet *et al*., 2006; Katavic *et al*., 2006) at room temperature. In this extraction procedure the one step urea (8M) treatment was done as described by Tzen *et al*. (1990) and Tzen and Huang (1992) which gave multiple bands (Fig 4. lane 2). As seen in the flow chart (Fig 1), apart from the one step urea treatment an additional step was carried out by keeping the urea concentration constant but altering the time of exposure (30 min, 1h and over night). Total oleosin was found to be 1.18% in 30 min exposure, 0.75 % in 1 h and 0.57 % over night exposure (Table 1). To confirm the purity of oleosin SDS-PAGE was done. Higher content of total oleosin resulted in multiple bands and as the time of exposure was increased the intensity of exogenous proteins decreased (Fig 4. Lane 3, 4 and 5). Thus we can assume that the one step urea treatment may have not removed many of the exogenous proteins which could lead to over estimation of total oleosin in *B. juncea*. The reduction in exogenous proteins after exposure to high salt concentration could be due to the breakage of salt bridges with phospholipids as explained by De Chirico et al. (2018).

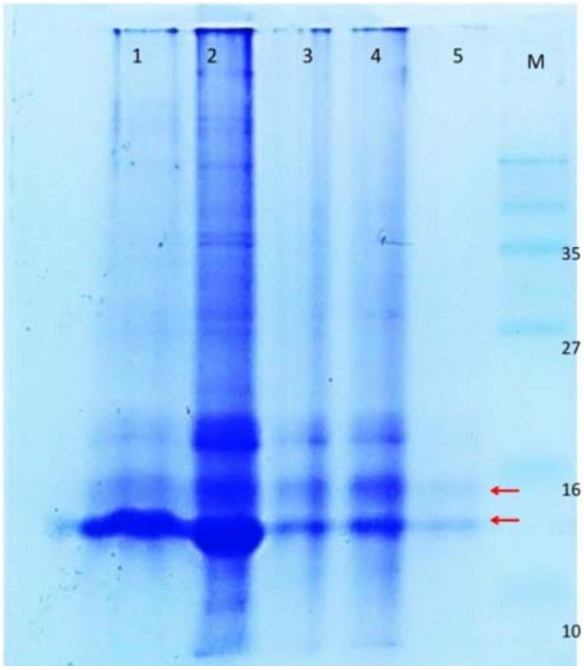

Fig. 4. SDS-PAGE profile of different OB protein extracted. Lane 1 Crude OB extract; Lane 2 Oleosin extract with one step 8M Urea treatment; Lane 3 Oleosin extract after one step urea treatment with an additional exposure time of 30min at RT; Lane 4 Oleosin extract after one step urea treatment with an additional exposure time of 1h at RT; Lane 5Oleosin extract after one step urea treatment with an additional exposure time of overnight at RT; Lane M-wide range protein ladder (GCC-Biotech).

Brassica seed has about 20-30 % protein on whole seed basis, the seed meal contains about 36-44% protein. Thus removal of all unwanted proteins for further understanding the oleosin is important. Our results also were in agreement with reports of De Chirico et al. (2018) in which the total oleosin content decreased with downstream purification. In this study total oleosin content ranged from 0.21% (HB9902) to 0.68 % (RL-1359). Although our observations were not at par with that of Tzen *et al*. (1993), it was within the range of 0.59 to 3.46% from varied number of species (Tzen *et al*. 1993), confirming that the overnight treatment ensures purity. The removal of exogenous proteins by exposing to high salt concentration could improve the solubility of proteins and removal of contaminant proteins, leaving behind only the integral ones (Arankawa and Timashaff, 1985; Millichip *et al*., 1996, De Chirico *et al*., 2018).

In SDS–PAGE the most prominent band was around 14KDa and 16 KDa that remained even after overnight urea treatment, showing that they are integral part of oil bodies. The molecular weight of oleosin has been reported to range from 14 to 30 KDa (Murphy 2001). Our extraction also showed oleosin molecular weight to be within this range and the extra bands could be its isoforms. According to Tzen *et al*. (1998) oleosin isoforms co-exist on the oil body surface and its presence could have physiological significance in regulating oil body mobilization and distribution of carbon resources of triacyglycerol during seed development.

Oleosin-oil body-oil content relationship

In this study the aim was to correlate oleosin content with the size of OB as reported in previous works (Hu *et al*. 2009, Siloto *et al*., 2006). It was observed that as the oleosin content decreases the size of OB increases (Fig 5). The findings were in agreement with Shimada *et al.* (2008) who observed inverse relationship between OB size and oleosin content. According to Hu *et al.* (2009), the number of small oil body could correlate with increase in oil content.

Studies in Arabidopsis showed mutation of gene encoding oleosin protein resulted in unusually large size OB (Siloto *et al*., 2006).Disruption of the gene encoding oleosin also leads to altered accumulation of lipids (Siloto *et al*., 2008). To understand this we did a comparative study between the OB size, oleosin content and oil content in which we observed that increase in oil content was due to the decrease in OB size which in turn was a result of increased oleosin content (Fig 5). It is probably the oleosin content that has a major role in maintaining the integrity of oil body there by influencing its size. The small size of OB would provide a large surface area per unit of stored lipids (e.g. Triacylglycerol), which could influence the binding of enzymes like lipases and facilitate lipolysis during germination.

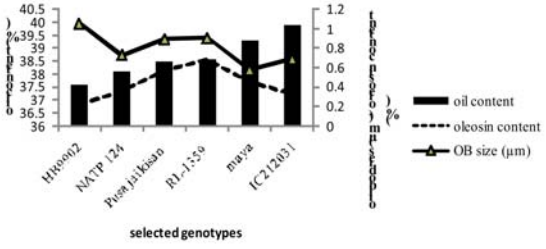

Fig. 5. A relationship overview of the selected genotypes with respect to oil content, oleosin and OB size

Conclusion

Measurement of oil body size was standardizedthe using simple compound light microscope also purification of oleosin was done by additional step of urea treatment with exposure time of overnight at room temperature resulting reduction in intensities of exogenous bands leaving only integral protein. The modified protocol could pave way for future studies on OB and its oleosin protein. This study on OB size and structure will help to further understand the mechanism of oil accumulation in *B. juncea* for increasing oil content.The stability of OB could be of interest to the food industries. Isolation of oleosin could add value to food industries for use in emulsifiers as they are good in stabilizing artificial emulsions.

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