ELSEVIER

Journal of Chromatography B, 706 (1998) 131–140

JOURNAL OF CHROMATOGRAPHY B

Characterization of the oligomeric behavior of a 16.5 kDa peanut oleosin by chromatography and electrophoresis of the iodinated form

Laurent Pons, Agnès Olszewski, Jean-Louis Guéant*

Laboratoire de Pathologie Cellulaire et Moléculaire en Nutrition, EP CNRS 0616, Faculté de Médecine, B.P. 184, 54505 Vandoeuvre-lès-Nancy Cedex, France

Abstract

Oleosins are amphipathic proteins associated with oil bodies in seeds. We purified the major 16 500 peanut oleosin by preparative SDS–PAGE. Autoradiography after SDS–PAGE separation of the iodinated oleosin revealed covalently bound oligomers with M_r of 21 000, 33 000, 44 000 and 51 000. The strong capacity of these oligomers to form aggregates and to be incorporated into large-sized detergent micelles was demonstrated by gel permeation and isoelectric focusing. A 50% ethanol concentration was necessary to elute the 16 500 oleosin from octyl groups in hydrophobic interaction chromatography showing its natural tendency to interact with lipid acyl chains. This oligomerization behavior in aqueous solution is an indirect reflection of the interactions that occur in the oil body. © 1998 Elsevier Science B.V.

Keywords: Oleosin; Acyl binding proteins; Peanut; Iodination; Oligomerization

1. Introduction

The storage of triacylglycerols in seeds [1–3] and pollen grains of plants [4] is confined to small, discrete spherical organelles called oil bodies [5,6]. These intracellular organelles contain a matrix of triacylglycerols surrounded by a half unit membrane of one phospholipid layer [2] embedded with abundant and unique proteins termed oleosins [7]. Oleosins are amphipathic and alkaline proteins [8,9] of small molecular mass, ranging from 15 000 to 26 000, depending on the isoforms and plant species in which they occur [5,6]. Structural studies using circular dichroism and Fourier transform infrared spectroscopy [10,11] combined with computer deduced analysis of the primary sequences [12,13] have given rise to a well established model of the oleosin secondary structure. Oleosins consist of three distinct structural domains, including an amphipathic N-terminal domain, a highly conserved central hydrophobic domain of 72 amino acid residues and an amphipathic α-helical domain at or near the C-terminus. The central stretch is likely to be the longest hydrophobic sequence found in any organism [5,6]. It is depicted as an antiparallel β-stranded domain [12,14] that penetrates the phospholipid layer into the triacylglycerol matrix. From this unique conformation, a meshwork of tightly-bound oleosin molecules is induced, covering the entire surface of the oil body, rendering it inaccessible to attack by proteases, phospholipases [15] or detergents [16] and preventing coalescence during tissue desiccation [17]. Therefore, the remarkable stability of the oil

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00530-6

body is the result of the strong tendency for oleosins to closely interact together.

Although pioneer works on oil bodies (spherosomes) [1,2] have been initiated from peanut seeds (*Arachis hypogaea* L.), peanut oleosins have been rarely approached in the literature [18,19]. The main study on peanut oil body membranes was realized by Jacks et al. [20]. These authors described that the preponderant component of lipid-free oil body membranes was proteinaceous and consisted of at least two polypeptides of 16 000 and 33 500, as resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The amphipathic and self interaction properties of oleosin in aqueous solution have not been studied until now. In the present study, we report the purification of a 16 500 peanut oleosin and the investigation of its hydrophobic and oligomerization properties in aqueous phase by isoelectric focusing (IEF) and chromatographic methods [size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC)].

2. Experimental

2.1. Chemicals

Carrier free [^{125}I]-iodine (specific activity 573.5 MBq/μg of I, 10 μl solution of 1 mCi) as Na[^{125}I] was purchased from Amersham International (Buckinghamshire, UK). Iodo-Beads Iodination Reagent and BCA Protein Assay Reagent were obtained from Pierce (Rockford, IL, USA). Chromatographic gels, Sephadex G25 medium (PD-10), Superose 12 prep grade, Octyl Sepharose 4 Fast Flow and carrier ampholytes for IEF (Pharmalyte, 40%, v/v stock solution) were obtained from Pharmacia Biotech (Uppsala, Sweden). Dialysis tubing was Spectra/Por with a molecular mass cut-off of 6000–8000 (Spectrum Medical Industries, Los Angeles, CA, USA). Organic solvents of analytical grade were purchased from Merck (Darmstadt, Germany). Urea, guanidine hydrochloride, SDS and CHAPS were of electrophoresis grade and supplied, as other currently used reagents, by Sigma (St. Louis, MO, USA).

2.2. Plant material

Mature peanut (*Arachis hypogaea* L., c.v. Virginia) seeds were obtained from a local grocery and stored at 4°C. Shelled cotyledons were freed from testae and embryonic axes, rinsed and then soaked in water for 1 h before use.

2.3. Isolation of oil bodies

Oil bodies were isolated by the centrifugation–flotation technique on a sucrose cushion developed by Huang [3]. The embryo tissue was homogenized at 4°C in grinding medium (5 g of tissue/20 ml) with an Ultra-Turrax T25 of 18 mm diameter (Ika-Labortechnik, Staufen, Germany) at high speed for 40 s. The grinding medium contained 0.6 *M* sucrose, 1 m*M* EDTA, 10 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* DTT and 0.15 *M* Tricine buffer adjusted to pH 7.5 with KOH. The homogenate was filtered through eight layers of cheesecloth.

Each 15-ml filtrate was placed at the bottom of a 30 ml Nalgene centrifuge tube (Nalge Company, Rochester, NY, USA) and 15 ml of flotation medium (grinding medium containing 0.4 instead of 0.6 *M* sucrose) was layered on top. The tube was centrifuged at 10 000 *g* for 30 min at 4°C (Model L5.50 Ultracentrifuge) in a swinging-bucket rotor (SW27 rotor) (Beckman Instruments, Fullerton, CA, USA). Oil bodies which formed a white pad at the surface of the supernatant were removed with a spatula and resuspended in 15 ml of grinding medium containing an additional 2 *M* NaCl in a 30-ml centrifuge tube. An overlay of 15 ml of floating medium (grinding medium containing 2 *M* NaCl and 0.25 *M* instead of 0.6 *M* sucrose) was introduced and the tube was centrifuged. The fat pad on top was removed, resuspended in 15 ml of grinding medium overlaid with 15 ml of flotation medium and again centrifuged. This last step of flotation–washing was repeated once.

The oil bodies on top were collected and dispersed in grinding medium to give a final concentration of approximately 100 mg of lipid/ml (w/v) by weighing the fatty preparation. This enriched oil body fraction consisted of more than 99% of lipids, the

second major component was proteinaceous but represented only 0.25%.

2.4. Delipidation of oil bodies using organic solvents

The defatting process of the isolated oil bodies was carried out according to Tzen and Huang [15]. The washed oil body preparation previously obtained (10 ml) was extracted three times with two volumes of diethyl ether enabling the removal of triacylglycerols, after centrifugation at 13 600 *g* for 4 min in a swinging-bucket rotor. The remaining ether was evaporated under a vacuum ventilator.

The aqueous layer, together with the interfacial materials, was treated with two volumes of chloroform–methanol (2:1, v/v) and centrifuged at 13 600 *g* for 4 min.

The lower chloroform layer (which contained phospholipids) and the upper methanol water layer were removed. The remaining interfacial materials (which contained precipitated proteins) were washed three times using the following procedure. They were mixed with 5 ml of distilled water and 15 ml of chloroform–methanol (2:1, v/v) and centrifuged. The interfacial materials were collected and the protein content was determined by the bicinchoninic acid (BCA) method [21] using bovine serum albumin (BSA) as standard.

2.5. SDS–PAGE

The proteins in the crude oil body fraction were resolved by SDS–PAGE according to the discontinuous buffer system of Laemmli [22]. An aliquot of the dried extract was solubilized in sample buffer [62.5 m*M* Tris–HCl buffer (pH 7.5) containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.004% bromophenol blue] and the mixture was heated for 3 min at 100°C. The electrophoresis system consisted of a 12.5% T and 4% T polyacrylamide [30% T acrylamide–Bis (29:1), Bio-Rad, Hercules, CA, USA] in the separating gel and stacking gel, respectively. Migration was performed at 150 V constant for 1.5 h using the Mini-Protean II system (Bio-Rad). After electrophoresis, the gel was stained with Coomassie blue R350 (PhastGel Blue R, Pharmacia).

2.6. Purification of the 16 500 oleosin by preparative SDS–PAGE

Purification of the 16 500 peanut oleosin was carried out in two preparative SDS–PAGE stages using the Bio-Rad Model 491 Prep Cell with a continuous elution system according to the manufacturer's instructions.

The dried crude oil body fraction (6 mg of total proteins) was resuspended in SDS–PAGE sample buffer, denatured at 100°C for 3 min and loaded onto the electrophoresis device. The electrophoresis was run at a constant current of 40 mA (150–240 V). Starting from the bromophenol blue dye front, fractions of 2 ml were collected at an elution rate of 0.4 ml/min using a peristaltic pump P-3 (Pharmacia). Elution was monitored by following absorbance at 280 nm using a UV absorbance detector, Model 117 (Gilson, Villiers-le-Bel, France). Eluted fractions of interest were analysed by SDS–PAGE, pooled, dialysed extensively for 48 h against 10 m*M* Tris–HCl buffer (pH 7.5) and concentrated by ultrafiltration on a 1000 MWCO membrane (Filtron Technology, Northborough, MA, USA) using an Amicon 8050 cell (Beverly, MA, USA). Protein purity was checked by SDS–PAGE with Coomassie blue staining as described in Section 2.5.

2.7. Iodination of the 16 500 oleosin

The purified 16 500 peanut protein was iodinated using Iodo-Beads reagent based on Markwell's method [23]. Two Iodo-Beads were washed twice with 1 ml of 20 m*M* Tris–HCl buffer (pH 7.4) and dried on a Whatman filter paper. The beads were placed into 200 μl of the same buffer containing 100 μCi of Na[^{125}I] (carrier free) for 5 min. The purified protein (20 μg) was then added, reaching a total volume of 300 μl. After 15 min incubation, the beads were discarded and 200 μl of 20 m*M* Tris–HCl buffer (pH 7.4) containing 0.15 *M* NaCl and 0.01% (w/v) NaI was added. The radioactive mixture was applied onto a 5.0×1.5 cm I.D. Sephadex G25 column (PD-10) eluted with the aforementioned buffer in pres-

ence of Blue Dextran 2000 (Pharmacia). Drops eluted with the dye front were collected and represented at least 5.4% of the applied radioactivity. The subsequent fraction was more accurately separated from unbound [^{125}I]-iodine by performing gel permeation on a Superose 12 column. Therefore, specifically protein associated [^{125}I]-iodine was estimated as 70% of the total radioactivity. The yield of labelling was finally brought to a value of 3.8%. Gamma-counting was performed on LKB Wallac 1261 MultiGamma, Gamma Counter (Turku, Finland) with a counting efficiency of 80%. The specific radioactivity was 115 Bq/pmol (0.19 μCi/μg) of proteins and the average number of iodine atoms introduced per molecule of oleosin was calculated as 0.0016.

2.8. IEF of the radioiodinated purified oleosin

IEF was performed in a 110 ml LKB column, type 8100-1 (LKB, Bromma, Sweden), packed with carrier ampholytes of pH values ranging from 3 to 10 (Pharmalyte), at the final concentration of 1% (v/v) in a 5–50% (w/v) sucrose gradient containing 5 *M* urea. The iodinated protein was treated or not by 1% (w/v) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} before its incorporation into the column. Migration was carried out at 5 mA constant current for 48 h at 15°C. After this period, the column was eluted into 1 ml fractions at a flow-rate of 1.0 ml/min. γ-Radioactivity and pH were measured in each fraction.

The major peaks of each IEF experiment were pooled and extensively dialysed against 10 m*M* Tris–HCl buffer (pH 7.5). After concentration by evaporation, each peak was resolved by SDS–PAGE, under the conditions described in Section 2.5. The gels were fixed, dried and exposed with X-OMAT AR film (Eastman Kodak Company, Rochester, NY, USA) for five days at −70°C.

2.9. Superose 12 gel permeation of the iodinated 16 500 oleosin

The labelled oleosin (1 ml) was injected into a Superose 12 column (molecular mass exclusion limit $2\cdot10^6$ and optimal separation range $1000–3\cdot10^5$; 30×1 cm I.D.) eluted with 20 m*M* Tris–HCl buffer (pH 7.4) at a flow-rate of 0.5 ml/min, using a Spectra Series P200 pump (Spectra-Physics, Fremont, CA, USA). The void volume elution position was determined by Blue Dextran 2000. The effluent was collected in 1 min fractions for 100 min and each fraction was γ-counted. In order to investigate the oligomerization properties of this oleosin, several eluents were prepared on the basis of the aforementioned buffer. Six different eluents were tested: Tris+0.7 *M* NaCl (Tris+NaCl); Tris+10% (v/v) ethanol (Tris+10% ethanol); Tris+50% (v/v) ethanol (Tris+50% ethanol); Tris+6 *M* guanidine hydrochloride (Tris+GnHCl); Tris+8 *M* urea (Tris+urea) and Tris+0.2% (w/v) SDS (Tris+SDS). All buffers were filtered through 0.45-μm type HV membrane (Millipore Corporation, Bedford, MA, USA) and vacuum degassed for 1 h before use. The column was thoroughly washed with five column volumes of Tris buffer between changes of solvent. Under these conditions, no radioactivity was detected in the effluent neither at the end of the washing procedure nor at the beginning of each new run. Before injection, the sample was incubated with the elution buffer tested for 30 min under shaking. The amount of radioactivity injected ranged from $4.5\cdot10^{-3}$ to 0.608 μCi in function of incubation with the different buffer solutions. Insoluble material was removed by centrifugation for 15 min at 10 000 *g*. In the case of elution with Tris+SDS buffer, labelled oleosin was dissolved in 20 m*M* Tris–HCl buffer (pH 7.4) containing 2% (w/v) SDS and 5% (v/v) β-mercaptoethanol and heated at 40°C for 30 min with periodic shaking, prior to chromatography. Each condition was tested in duplicate. The recovery of radioactivity after each elution cycle was estimated at about 74% and was of the same magnitude whatever the amount injected or eluent used.

2.10. Octyl-Sepharose chromatography of the iodinated oleosin

The Octyl-Sepharose column (5×1 cm I.D.) equilibrated in 20 m*M* Tris–HCl buffer pH 7.4 at a flow-rate of 0.5 ml/min was loaded with 100 μl of [^{125}I]-oleosin. Fractions of 0.5 ml were then collected for 50 min and γ-radioactivity counted. After this loading step, the six buffers previously tested in gel permeation were used to desorb the immobilized

radioactivity. Between each change of eluent, the column was rinsed with five volumes of 20 m*M* Tris–HCl buffer (pH 7.4) and the γ-radioactivity counted to ensure that no [^{125}I]-oleosins were lost during the washing procedures.

3. Results

3.1. Purification of the 16 500 peanut oleosin

After the four centrifugation–flotation cycles on a sucrose cushion and thorough delipidation using diethyl ether and chloroform–methanol, the protein content in the dried extract was estimated at 0.1% (±0.02) of the seed mass. SDS–PAGE analysis with Coomassie blue staining of the crude oil body extract revealed a major enrichment in a 16 500 protein among 15 500 to 82 000 proteins with other 2 noticeable bands at 33 000 and 42 000 (Fig. 1, left). This 16 500 protein represents 53% of the total oil body membrane proteins as evaluated by densitometric analysis of the SDS–PAGE separated proteins. Because its high content, the 16 500 protein band was most likely to be the authentic oleosin rather than the minor contaminants. Two cycles of preparative SDS–PAGE were necessary to separate the 16 500 oleosin from its nearest contaminants of 16 000 and 15 500. The purified proteins were at least 99% pure as judged by SDS–PAGE (Fig. 1 left). When more purified protein quantities were applied to the gel, a slightly diffuse band at 33 000 clearly appeared.

In an endeavour to conciliate the extremely consuming protein amounts required for physicochemical characterization experiments with a highly sensitive method of detection used for studying the oligomeric behavior of peanut oleosins, we chose to label the 16 500 purified protein with [^{125}I]-iodine.

After radioiodination, autoradiography of the resolved SDS–PAGE purified oleosin consisted of two pronounced bands at 16 500 and 21 000 and several less marked bands at 33 000, 44 000 and 51 000 (Fig. 1, right).

3.2. IEF of the radioiodinated 16 500 oleosin

In IEF, the radiolabelled oleosin was resolved into acidic peaks with a main isoelectric point (p*I*) at 4.5 (Fig. 2A). When treated with 1% CHAPS, a zwit-

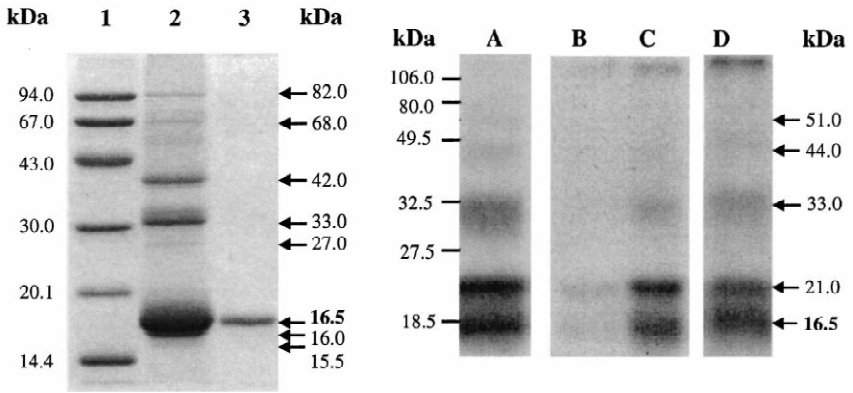

Fig. 1. SDS–PAGE analysis of the purification process (left). Electrophoresis was performed on a 12.5% T polyacrylamide gel and run at 150 V constant voltage. Protein bands were detected by Coomassie blue staining. Lane 1 : low-molecular-mass standards (Pharmacia), phosphorylase B (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400). Lane 2 : crude oil body extract (15 μg). Lane 3 : purified 16 500 oleosin (2.5 μg). Autoradiography after SDS–PAGE analysis of the iodinated oleosin before and after IEF (right). On the left side are positioned the low-range prestained standards (Bio-Rad), phosphorylase B (106 000), bovine serum albumin (80 000), ovalbumin (49 500), carbonic anhydrase (32 500), soybean trypsin inhibitor (27 500), lysozyme (18 500). Lane A : iodinated purified 16 500 oleosin. Lanes B and C: peaks of p*I* 2.8 and 4.5, respectively, of the isoelectrofocused oleosin (IEF A). Lane D : peak of p*I* 5.1 of the isoelectrofocused oleosin treated with 1% (w/v) CHAPS (IEF B). Whatever the peak collected from the IEF liquid column, the iodinated 16 500 oleosin was always resolved into similar oligomeric forms. This was observed both with and without addition of CHAPS detergent.

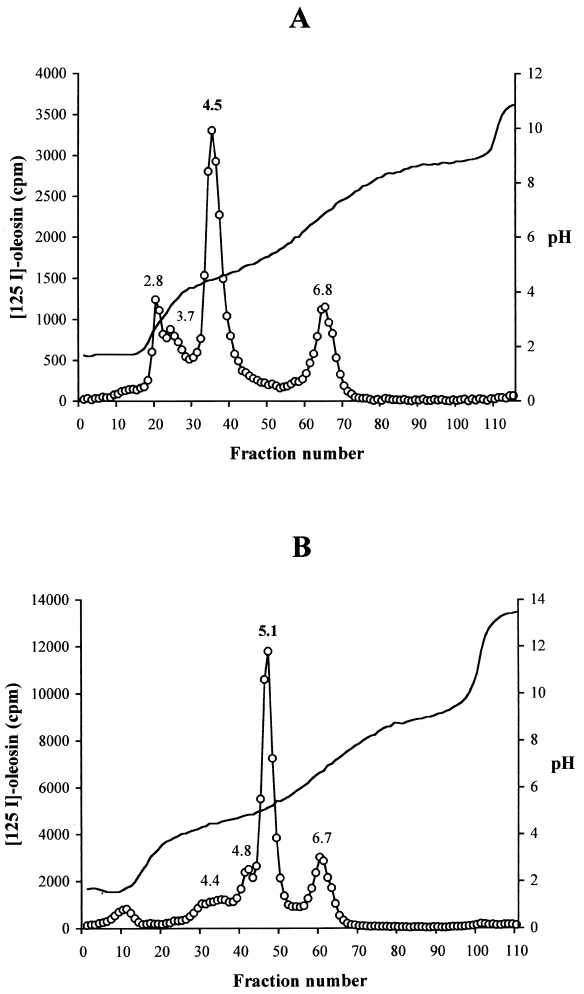

Fig. 2. IEF of the radioiodinated oleosin. IEF was carried out in a 110 ml LKB column, packed with carrier ampholytes of pH values ranging from 3 to 10 (Pharmalyte) at a final concentration of 1% (v/v) in a 5 to 50% (w/v) sucrose gradient containing 5 *M* urea. The iodinated protein was treated (0.065 μCi) (B) or not (0.027 μCi) (A) by 1% (w/v) CHAPS before its incorporation into the column. IEF was performed at 5 mA constant current for 48 h at 15°C. Each collected fraction (1 ml) was counted for radioactivity (○) and the pH measured (—). The [^{125}I]-oleosin was resolved essentially into acidic peaks. When CHAPS was added minor acidic peaks were reduced and the main p*I* shifted from 4.5 to 5.1.

terionic detergent, prior to IEF, the small acidic peaks disappeared and the main one shifted to a p*I* value of 5.1 (Fig. 2B). Another peak at a p*I* near neutrality was detected in both experiments. The acidic p*I* values correspond to aggregates of the unitary 16 500 oleosin as attested by the identical autoradiographic patterns obtained after SDS–PAGE separation (Fig. 1, right). The same relative intensity of each resolved band was also observed. An attempt to analyze the neutral p*I* peak by SDS–PAGE was unsuccessful since no more radioactivity was retained after dialysis. This indicated that the neutral product had an apparent molecular mass below 6000. This oleosin still exists as aggregates even under the combined effect of 5 *M* urea and detergent.

3.3. SEC of the 16 500 iodinated oleosin

In gel permeation, the radioiodinated oleosin was systematically eluted as an aggregate of high-molecular mass (M_r larger than $2 \cdot 10^6$) in the exclusion volume of the column (Fig. 3). However, under low-ionic strength elution conditions, an oligomer appeared at a retention time (t_R) of 35 min (approximately 44 000) (Fig. 3A). Radioiodinated intrinsic factor, a 44 000 soluble protein, was used as a standard and was eluted at the same retention time. When increasing the ionic strength of the mobile phase by addition of 0.7 *M* NaCl or lowering polarity by elution in presence of 10% or 50% ethanol, this oligomer disappeared (Fig. 3B). Strong chaotropic agents such as 8 *M* urea or 6 *M* guanidine hydrochloride induced minor dissociation of the aggregate into a compound eluted at a t_R of 44 min which may well be the monomeric 16 500 oleosin (Fig. 3C). When treated and eluted in presence of SDS, oleosin aggregates and eventually dissociated oligomers and monomers were included into large sized detergent micelles and were eluted in the exclusion volume as well as at a t_R of 27 min (Fig. 3D).

3.4. HIC of the 16 500 iodinated oleosin

Using HIC, 90% of the applied iodinated oleosin was adsorbed on octyl groups under low-ionic strength conditions. Very strong and exclusive hydrophobic interaction existed between the alkyl chain and the oleosin, since none of the eluents tested in gel permeation managed to desorb the protein. Only Tris–HCl buffer+50% ethanol succeeded in desorbing 54% of the totally bound radioactivity (Fig. 4).

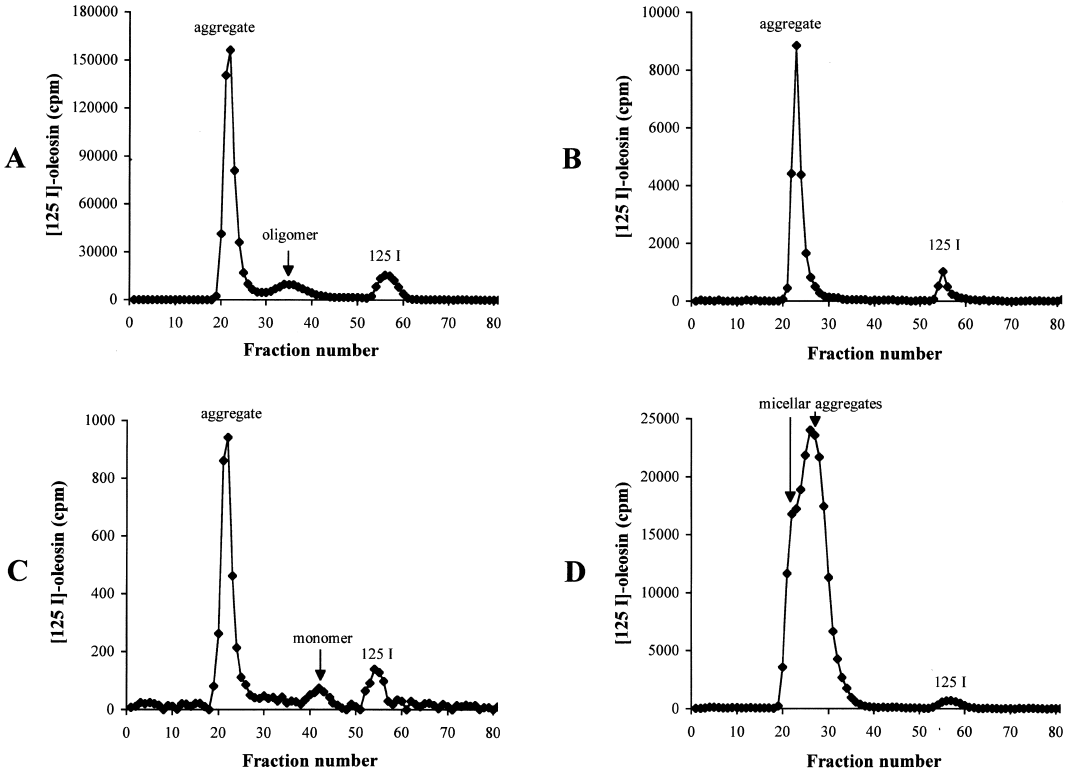

Fig. 3. Superose 12 gel permeation of the iodinated 16 500 oleosin. The Superose 12 column (30×1 cm I.D.) was eluted at a flow-rate of 0.5 ml/min. The exclusion volume was determined by the addition of Blue Dextran 2000. The effluent was collected into fractions of 0.5 ml volume which were γ-counted. Several amounts of radiolabelled oleosin were injected according to the different mobile phases: 0.608 μCi with 20 m*M* Tris–HCl buffer (pH 7.4) (A), 0.022 μCi with Tris buffer+0.7 *M* NaCl (B), $4.5 \cdot 10^{-3}$ μCi with Tris buffer+8 *M* urea (C) and 0.169 μCi with Tris buffer+0.2% (w/v) SDS (D). Before injection (1 ml), the sample was incubated with the tested elution phase for 30 min. In the case of elution with SDS, the sample was also heated at 40°C. Each condition was tested twice. Partial dissociation of the aggregate was obtained either at low ionic strength or in the presence of a high concentration of urea. Treatment and elution with SDS partially decreased the size of the aggregate, increasing the t_R to 27 instead of 23 min (void volume).

4. Discussion

In mature peanut seed, the major constituent (53%) of the oil body membrane associated proteins was made up of a single polypeptide with a molecular mass of 16 500 (Fig. 1). Early attempts to purify the oleosin from rapeseed [24] by ion-exchange or gel permeation chromatography were unsuccessful since, even in its solubilized form, the protein was present in large mixed aggregates with other polypeptides. Other methods using reversed-phase high-performance liquid chromatography (HPLC) failed to separate the 19 000 sunflower oleosin from its nearest contaminants [25]. Because of its accurate resolution combined with denaturing conditions, preparative SDS–PAGE has proved to be the method of choice in oleosin purification [24,26,18,8]. Two cycles of preparative SDS–PAGE were necessary to purify to homogeneity the 16 500 peanut oleosin (Fig. 1).

Oleosins are hydrophobic proteins which are aligned at the lipid–water interface. They are therefore quite different to soluble proteins and to the integral membrane proteins as regards the constraints that determine their secondary structure and their subsequent interactions. In solution, they show a strong tendency to oligomerize, even under strong denaturing conditions [11]. The proclivity of the 16 500 oleosin for self-association was latent as a 33 000 dimer appeared after increasing the protein

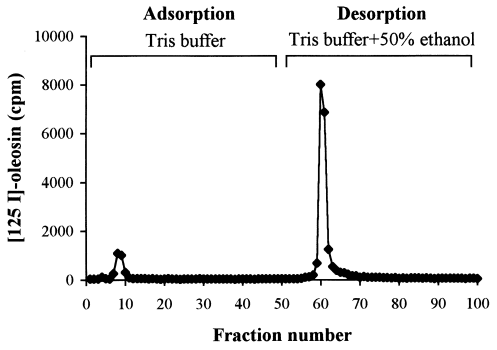

Fig. 4. Octyl-Sepharose chromatography of the iodinated oleosin. The Octyl-Sepharose column (5×1 cm I.D.) was loaded with [^{125}I]-oleosin (0.022 μCi) at a flow-rate of 0.5 ml/min in Tris–HCl buffer (pH 7.4). The same eluents as in gel permeation were used to desorb the bound protein (example shown: Tris buffer+50% ethanol). Between each change of mobile phase, a washing procedure was carried out with Tris buffer. Fractions of 0.5 ml were collected for 50 min and γ-radioactivity counted. The adsorbed [^{125}I]-oleosin was mainly eluted with 50% ethanol, demonstrating its high hydrophobicity.

quantities applied onto gel electrophoresis. The same phenomenon was observed with the 19 000 rapeseed oleosin which dimerized into a 40 000 band in SDS–PAGE [11]. To further explore this oligomeric behavior, we radiolabelled the purified protein with [^{125}I]-iodine. Iodo-Beads oxidizing agent consists of chloramine-T derivatized polystyrene beads which provide relatively mild and controllable conditions for protein iodination [23]. Undesirable side chain reactions and aggregation frequently induced by soluble chloramine-T [27–29] was circumvented by the use of this solid-phase method [30]. It can be assumed that iodination of this oleosin induced mild denaturation since as low as 0.0016 iodine atoms were introduced per molecule of protein. Equivalent ratios of iodination have proved to be non-denaturing for other proteins such as albumin and fibrinogen [28]. In our laboratory, iodination of BSA and β-lactoglobulin was achieved without leading to artificially induced aggregates or spurious behavior in gel permeation and SDS–PAGE or to loss of their binding capacity [31]. Although these proteins are soluble in aqueous phase, they are well known to oligomerize under specific conditions [32,33]. Nevertheless, in the case of the 16 500 peanut oleosin, covalently bound oligomers were formed after iodination as attested by autoradiography after SDS–PAGE separation (Fig. 1). Concomitantly to the monomeric 16 500 oleosin, a 33 000 and 51 000 band appeared which could correspond to dimeric and trimeric associations, respectively. Furthermore, another marked band at 21 000 was observed with its possible dimeric form at 44 000. This second band is quite surprising both in itself and in its intensity but may be hypothesized as the association, during the labelling process, between the monomeric oleosin and an unidentified peptide of approximately 4500 (contaminant or degradative products derived from the 16 500 oleosin). During electrophoresis, in order to increase the resolution, we let the bromophenol blue dye front leave the bottom of the gel. For this reason, no lower sized bands below 16 500 were noticed. However, shorter running time electrophoresis revealed a labelling, after autoradiography, at the level of the front dye or just behind it. This could correspond to unresolved small peptides as free [^{125}I]-iodine was eliminated by gel permeation.

A discrepancy in the behavior of the iodinated oleosin was observed between gel permeation and SDS–PAGE. In the former technique, labelled oleosins were systematically eluted as high-molecular-mass aggregates (M_r of more than $2 \cdot 10^6$). When playing on the elution conditions, not more than 10% of these aggregates were dissociated into oligomeric or monomeric forms (Fig. 3). Under low-ionic strength conditions, an oligomer at approximately 44 000 appeared which was absent when 0.7 *M* NaCl was added to the mobile phase. Chaotropic agents such as urea and guanidine hydrochloride, induced a minor dissociation into a monomeric compound. If the same denaturing treatment as SDS–PAGE was applied on the proteins, oligomers and monomers were included into large sized micelles which were eluted with the void volume and at a t_r of 27 min, respectively. Change in the polarity of the eluent phase (10 to 50% ethanol) did not alter the integrity and therefore the elution profile of the aggregates. On the contrary, in SDS–PAGE, no particular labelling was noticed at the bottom of the wells or at the entry of the running gel, pointing out that the high-molecular mass aggregates were disrupted and resolved into subunits of medium sizes, ranging from 16 500 to 51 000. Hence, covalently bound oligomers are able to self associate into larger aggregates. These aggregates are very stable, as proved by the

difficulties for harsh denaturants to dissociate them, in SEC. This aggregation at a higher level seems to implicate strong non-covalent interactions as attested by the weak influence of ionic strength and urea or guanidine hydrochloride.

In IEF, the same main bands were observed after autoradiography of the SDS–PAGE resolved main IEF acidic peaks (Fig. 1). Surprisingly, when CHAPS was added, the microheterogeneity of the acidic peaks was reduced in favor of the main peak, which was shifted to a slightly higher p*I* value (Fig. 2). Hence, oligomers associated with CHAPS to form stabilized micelles with a p*I* of 5.1. The smaller neutral peak may correspond to peptides as no radioactivity remained after dialysis. Addition of CHAPS did not influence the p*I* of this peak. No basic peak corresponding to the monomeric form was observed.

It seems, therefore, that it is the state of the purified oleosin, in aqueous solution, which induced specific reactions with chloramine-T leading to the formation of covalently bound oligomers. This very particular type of chemical cross-linking, mediated by iodination, might occur only with hydrophobic proteins constrained in an unfavourable medium (aqueous phase) to tightly self-interact. Under these prerequisite conditions, groups on proteins held in close proximity and appropriate orientation by the folded conformation may undergo reactions which are not significant between separate soluble molecules when in solution. A similar behavior was observed with membrane proteins of the erythrocyte which are frequently submitted to oxidative stress [34]. The chemical nature of the protein cross-linking may be questioned. The fact that β-mercaptoethanol failed to dissociate the oligomers, suggests that they do undergo covalent, but not disulfide bonding. Moreover, cysteine residues are almost absent in the composition of oleosins [7,9,25] and especially peanut oleosins [20]. Thus, it may be interesting to mention the covalent cross-link between Glu 35 and Trp 108 of hen lysozyme mediated by iodine treatment [35] as a possible explanation of what takes place between peanut oleosins during iodination.

In HIC, the high adsorption percentage (90%) of the iodinated oleosin indicates that although mainly soluble, the aggregates still exhibit hydrophobic domains. Only an eluent containing 50% ethanol succeeded in desorbing merely half of the immobilized radioactivity (Fig. 4). This seems consonant with the natural role of oleosins in the oil body which is to closely interact, by means of its highly hydrophobic central stretch, with the triacylglycerol matrix and also, with the aliphatic chains of phospholipids. The choice of octyl rather than phenyl solid-phase was therefore more adequate for evaluating the binding activity of the protein to acyl chains.

Oleosins act as emulsifying agents at the oil–water interface. Such proteinaceous emulsifying agents are of great interest since they can stabilize micellar preparations and be used for the vectorized delivery of drugs or antisens oligonucleotides.

In conclusion, we purified to homogeneity a 16 500 peanut oleosin. This protein showed a striking oligomerization capacity, even under drastic denaturing conditions as observed by IEF, gel permeation and HIC after iodination. However, this behavior is in accordance with its function which is to stabilize the oil body, the triacylglycerol storage organelle in plants.

Acknowledgements

We are grateful to the Institut Danone for its financial support during this study.

References

[1] T.J. Jacks, L.Y. Yatsu, A.M. Altschul, Plant Physiol. 42 (1967) 585.
[2] L.Y. Yatsu, T.J. Jacks, Plant Physiol. 49 (1972) 937.
[3] A.H.C. Huang, in H.F. Linskens and J.F. Jackson (Editors), Modern Methods of Plant Analysis, Springer Verlag, Berlin, 1985, Ch. 1, p. 145.
[4] R.G. Stanley and H.F. Linskens, Pollen, Springer Verlag, Berlin, 1974, p. 221.
[5] A.H.C. Huang, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 177.
[6] A.H.C. Huang, Plant Physiol. 110 (1996) 1055.
[7] R. Qu, A.H.C. Huang, J. Biol. Chem. 265 (1990) 2238.
[8] R. Qu, S.M. Wang, Y.H. LIN, V.B. Vance, A.H.C. Huang, Biochem. J. 235 (1986) 57.
[9] J.S. Keddie, E.W. Edwards, T. Gibbons, C.H. Shaw, D.J. Murphy, Plant Mol. Biol. 19 (1992) 1079.

[10] M. Li, L.J. Smith, D.C. Clark, R. Wilson, D.J. Murphy, J. Biol. Chem. 267 (1992) 8245.
[11] M. Li, J.S. Keddie, L.J. Smith, D.C. Clark, D.J. Murphy, J. Biol. Chem. 268 (1993) 17504.
[12] J.T.C. Tzen, G.C. Lie, A.H.C. Huang, J. Biol. Chem. 267 (1992) 15626.
[13] V.B. Vance, A.H.C. Huang, J. Biol. Chem. 262 (1987) 11275.
[14] C. Ratnayake, A.H.C. Huang, Biochem. J. 317 (1996) 956.
[15] J.T.C. Tzen, A.H.C. Huang, J. Cell Biol. 117 (1992) 327.
[16] D.J. Murphy, I. Cummins, Phytochemistry 28 (1989) 2063.
[17] D.J. Murphy, Prog. Lipid Res. 32 (1993) 247.
[18] J.T.C. Tzen, Y.K. Lai, K.L. Chan, A.H.C. Huang, Plant Physiol. 94 (1990) 1282.
[19] J.T.C. Tzen, Y.Z. Cao, P. Laurent, C. Ratnayake, A.H.C. Huang, Plant Physiol. 101 (1993) 267.
[20] T.J. Jacks, T.P. Hensarling, J.N. Neucere, L.Y. Yatsu, R.H. Barker, J. Am. Oil Chem. Soc. 67 (1990) 353.
[21] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Malia, F.H. Gartner, M.D. Proverzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[22] U.K. Laemmli, Nature 227 (1970) 680.
[23] M.A.K. Markwell, Anal. Biochem. 125 (1982) 427.
[24] D.J. Murphy, I. Cummins, Plant Sci. 60 (1989) 47.
[25] M. Millichip, A.S. Tatham, F. Jackson, G. Griffiths, P.R. Shewry, A.K. Stobart, Biochem. J. 314 (1996) 333.
[26] D.J. Murphy, I. Cummins, A.S. Kang, J. Sci. Food Agric. 48 (1989) 209.
[27] K. Krohn, L. Sherman, M. Welch, Biochim. Biophys. Acta 285 (1972) 404.
[28] J. Lee, R.E. Coleman, L.A. Sherman, J. Lab. Clin. Med. 89 (1977) 836.
[29] R.H. Seevers, R.E. Counsell, Chem. Rev. 82 (1982) 575.
[30] D.S.C. Lee, B.W. Griffiths, J. Immunol. Methods 74 (1984) 181.
[31] A. Mansouri, J.L. Guéant, J. Capiaumont, P. Pelosi, P. Nabet and T. Haertlé, Biofactors, (1997) in press.
[32] E.M. Brown, R.J. Carroll, P.E. Pfeffer, J. Sampugna, Lipids 18 (1983) 111.
[33] M. Katakam, A.K. Banga, J. Pharm. Pharmacol. 47 (1995) 103.
[34] E. Yang, W.H. Huestis, J. Biol. Chem. 269 (1994) 14518.
[35] T.E. Creighton, Proteins: Structures and Molecular Properties, W.H. Freeman and Company, New York, 1993, Ch. 7, p. 275.