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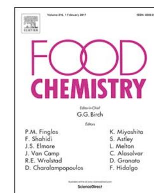

Enhancing the recovery of oilseed rape seed oil bodies (oleosomes) using bicarbonate-based soaking and grinding media

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ABSTRACT

An aqueous process for the recovery of oil bodies from rapeseed using sodium bicarbonate-based soaking and grinding media (pH 9.5) was investigated. The effect of the ratio between seed and mass of media during grinding and molarity of the medium used on oil body integrity, purity and storage stability have been studied. The grinding of seeds in solution at a ratio of 1:7 (w/w) significantly improved the quality of oil body suspension to a size more in-line with that seen in vivo (average $D_{4,3}$ of 1.19 µm). The purity and the composition of the recovered oil bodies depends on the molarity of medium used; the use of a sodium bicarbonate solution (pH 9.5, 0.1 M) in the grinding and washing steps produced oil body preparations with the same purity as that resulting from washing a crude preparation with 9 M urea. The resultant emulsion had improved physical stability over a storage period of one month.

1. Introduction

Oil bodies (OBs, denoted often also as oleosomes or lipid bodies) provide the seed with energy during germination; they are generally between 0.2 and 2.5 µm in diameter (Tzen & Huang, 1992). When viewed under an electron microscope, an electron-opaque matrix of triacylglycerols (TAGs) surrounded by one electron-dense layer of phospholipids (Tzen & Huang, 1992; Yatsu & Jacks, 1972) with embedded surface active proteins (*e.g.* oleosin) can be seen (Tzen, Cao, Laurent, Ratnayake, & Huang, 1993). The oleosin contains three basic structural domains: an amphipathic domain present at the N-terminal and C-terminal, residing on the OB surface ensuring stability *via* steric hindrance and electronegative repulsion, and a highly hydrophobic domain penetrating into the TAG matrix (Frandsen, Mundy, & Tzen, 2001; Murphy & Cummins, 1989; Qu & Huang, 1990). The unique structure of the oleosin is thought to prevent coalescence of OBs both in the cytosol of oilseed cells and *ex vivo* (Tzen & Huang, 1992).

The conventional process of oil extraction from oleaginous seeds using organic solvents and the subsequent oil refining has a high environmental impact, and it is hazardous due to flammability and risk of explosion. During this process, OBs are destroyed as the neutral oil (triacylglycerol) partitions into the organic solvent. The production of oil from vegetable sources is estimated to be approximately 150 million tonnes per annum, of which 30% is processed into emulsions using high energy homogenisation and the addition of emulsifiers/surface active agents (Gunstone, 2004). Many natural and processed foods are considered either partly or wholly as oil-in-water emulsion (Mcclements, 2004). When OBs are released (recovered) from the cells of oilseeds into an aqueous medium (a relatively benign wet milling process) they may form a stable oil-in-water emulsion, depending on the solution implied. Compared with conventional oilseed processing, the recovery of OBs as a natural source of pre-emulsified oil, to be used in a range of food applications, is likely to reduce the impact on the environment. OBs have been recovered from a number of different seeds, such as pumpkin (Adams et al., 2012), soybean (Chen, Mcclements, Gray, & Decker, 2012; Chen & Ono, 2010; Iwanaga, Gray, Decker, Weiss, & Mcclements, 2008), and maize (Nikiforidis & Kiosseoglou, 2009). Despite the differences between the authors, the key points for the extraction of OBs are similar, comprising: disruption of the seed matrix in aqueous media; collection of a "crude" OB cream after centrifugation and washing of the cream. The latter stage is aimed to remove the polysaccharide, seed debris and exogenous proteins that are not covalently bonded to the OB (Tzen, Peng, Cheng, Chen, & Chiu, 1997), using buffers, detergents or chaotropic agents, to obtain a purified OB cream free from exogenous material. As detailed by Millichip et al. (1996) urea (9 M) is a rigorous washing agent that produces an OB preparation free from exogenous seed proteins without destabilising the integral oleosin proteins necessary to retain OB integrity (Lacey, Wellner, Beaudoin, Napier, & Shewry, 1998).

Some studies have identified enzymes associated with recovered oil

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bodies (Chen & Ono, 2010; Chen, Zhao, Cao, Kong, & Hua, 2014; Karkani, Nenadis, Nikiforidis, & Kiosseoglou, 2013; Zhao, Chen, Chen, Kong, & Hua, 2016). The carry-over of enzyme activity (proteolytic or lipolytic) could cause destabilisation of OBs ex-vivo. The removal of these enzymes would therefore reduce the temperature and time required for effective biochemical stabilisation of the OB preparation. Urea is not a food grade material, so cannot be used to wash OBs and remove enzymes. However, as the enzymes contain multiple charged groups, they can be solubilised in salt solutions, and their solubility can increase with increasing salt concentration, in a phenomenon called "salting-in" (Arakawa & Timasheff, 1985). We hypothesised that a salt such as sodium bicarbonate, could be a suitable food grade replacement of the urea. The aim of this work was to test the ability of a sodium bicarbonate solution (pH 9.5) to recover intact OBs with reduced carry-over of exogenous proteins. The effect of changing the molarity of the bicarbonate solution, and the ratio between seed mass and mass of the medium during grinding were tested.

2. Materials and methods

Oilseed rape seeds (variety Quartz) were sourced from a local farm at time of harvest (2015), and stored at 20 °C and rH 50% until use. All chemicals and reagents were purchased from Sigma-Aldrich Trading Co., Ltd. of analytical reagent grade or higher. Precision Plus Protein Dual Color Standard (Bio-Rad, USA) was used as protein marker. Ultrapure water (Nanopure Infinity system, Barnstead International, IA) was used for all media.

2.1. Oil body recovery and purification

OBs were isolated from oilseed rape seeds and purified according to the method of Tzen et al. (1997) with the following modifications. Seeds (200 g) were soaked in sodium bicarbonate pH 9.5 (0.01, 0.1 or 0.3 M, adjusted with 0.1 M NaOH) or water, at ratio of 1:4 (w/w) at 4 °C for 16 h (unpublished data) and the soaking medium was then discarded. The soaked seeds were then ground in the same medium type as used on soaking (4 °C) at ratios (dry seed weight based) of 1:4 (w/w) or 1:7 (w/w) in a Kenwood blender (BLX52) at full power for 90 s. After grinding, the slurry was left stirring for 24 h at 4 °C, to improve the yield of oil bodies, and then filtered through three layers of cheesecloth. The filtrate was transferred in 400 mL tubes and centrifuged at 10000*g* for 30 min at 4 °C using a Beckman J2-21 centrifuge, fixed rotor JA-10. The upper layer was isolated using a spatula, drained on filter paper (Whatman, grade 5) and called crude oil body fraction (COB). Sodium azide (0.02 mM) was added to all media to avoid microbial spoilage. The COB preparation was dispersed in washing solution (0.1 M $NaHCO_3$, pH 9.5 or 9 M urea, 1:4 w/w) and centrifuged (10000*g*, 30 min). To remove any residue of the washing solution, the fat pad was isolated using a spatula, drained on filter paper, suspended in water (1:4 w/w) and centrifuged (10000*g*, 30 min). After centrifugation, the cream layer was collected and designated as urea-washed oil body cream (Urea-WOB) or sodium bicarbonate washed oil body cream ($NaHCO_3$-WOB). All creams were stored at 4 °C and analyses were completed within 24 h.

2.2. Particle size measurement

The particle size of OB suspensions was measured with a LS 13320 laser diffractometer (Beckman-Coulter, USA) using the Mie theory of the scattering of light by spherical particles. The samples were diluted as appropriate prior to measurement. The real part of the refractive index, was 1.462, corresponding to the refractive index of rapeseed oil. The imaginary part, corresponding to the 'attenuation coefficient' that describes the turbidity of a sample, was set to 0.01 according to the laser diffractometer guidelines for lightly coloured translucent materials. For the description of particle size distribution (PSD), the volume frequency distribution (%/µm) and the volume mean diameter ($D_{4,3} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$) have been reported. The fraction of intact droplets (≤ 2.5 µm in diameter), expressed as percentage, was found using the LS 13320 software from the cumulative volume frequency distribution curve.

2.3. Preparation of oil body emulsions for stability test

Oil body emulsions were prepared suspending the washed cream in water (10% lipid weight basis) using a vortex at maximum speed (1 min). Sodium Azide (0.02 mM) was present in all emulsions to avoid microbial spoilage. The $D_{4,3}$ was measured at time 0, 7, 14, 21 and 28 days storing the samples at 20 °C.

2.4. Lipid extraction

COB or WOB creams (approximately 0.2 g) were dried in a vacuum oven (40 °C, −900 mbar) for at least 48 h until constant mass. Dry matter was ground (mini-bead beater, 3450 rpm) for 2 min in isooctane (1 ml) to extract the oil (Gray, Payne, Mcclements, Decker, & Lad, 2010). Samples were centrifuged (Thermo Heraeus Fresco 21) at 17,000*g* for 5 min at 4 °C and the upper phase was removed with a pipette; this process was repeated three times and the recovered lipids dried under nitrogen. The resultant pellet after delipidation was used for protein extraction and analysis as described in Section 2.6.

2.5. Protein content

The proteins were extracted from delipidated dry material (as detailed in Section 2.4) using 2% sodium dodecyl sulfate solution (SDS, 1 ml) and incubated for 30 min at 60 °C in a water bath. Protein extracts were vortexed for 1 min and centrifuged at 17000*g* (5 min, 4 °C). The supernatant was collected and assayed on the same day for protein content using BCA assay (Smith et al., 1985). On performing the analysis, samples were diluted to obtain absorbance values within the range of the standard curve.

2.6. SDS-PAGE

To compare the degree of exogenous protein carried over in the extracts, protein samples were appropriately diluted in 2% SDS solution normalised by the amount of oleosin. 20 µL of protein extract was then mixed with an equal volume of sample buffer (Laemmli buffer (Biorad, UK) + 5% β-mercaptoethanol), heated at 95 °C for 5 min and then cooled on ice. Proteins were resolved by SDS-PAGE using 4–15% polyacrylamide gels (Mini-Protean TGX Gels, 10-well, 50 µL, Bio-Rad, Hercules, USA); gels were positioned within a SE 600 BioRad separation unit and suspended in tank buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, pH 8.3). Electrophoresis was run at 100 V for 40 min. After electrophoresis, the gels were washed three times (10 min, H_2O). The gel was then stained using comassie brilliant blue R-250 (1 h) and de-stained with de-staining solution (2 h). Gels were imaged using Bio-Rad Gel Doc XR System.

2.7. Zeta potential measurement

A series of OB emulsions were prepared in ultrapure filtered (0.2 µm Millex syringe filter unit, Millipore) water at a concentration of 0.008% (lipid weight basis) and the pH adjusted between 3 and 10 using 0.1 M HCl or 0.1 M NaOH solutions. After stabilization of the pH, the emulsions were injected into the measurement chamber of the particle electrophoresis instrument (Delsa Nano C Particle Analyser, Beckman Coulter, Inc., USA). The instrument settings used were: temperature = 25 °C; refractive index of dispersant = 1.333; viscosity of dispersant = 0.891 mPa s; relative dielectric constant of dispersant = 79.0; electrode spacing = 50.0 mm; voltage = 35 V. The ζ-

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