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Elucidation of the role of oleosin in off-flavour generation in soymeal through supercritical CO_2 and biotic elicitor treatments

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ABSTRACT

Defatting soybean by sophisticated oil extraction method utilising supercritical CO_2 resulted in a significant decrease in the residual phospholipids (PLs) compared with soymeal obtained by conventional cold percolation method utilising hexane as the extraction solvent. Interestingly, the levels of residual PLs showed a proportionate relationship with thiobarbituric acid (TBA) number, an indicator of lipid peroxidation responsible for off-flavour generation. Furthermore, two oleosins (18 and 24 kDa) were isolated from the oil bodies extracted from soybean seeds and positively characterised for phospholipase A_2 (PLA_2) activity, suggesting their plausible involvement in off-flavour generation in soymeal. The treatment of soybean seeds, before oil extraction, with different concentrations of biotic elicitors such as chitosan and jasmonic acid also significantly reduced the levels of residual PLs as well as the TBA number. The biotic elicitor treatment could thus prove to be an important strategy for the reduction of off-flavour in protein-rich soymeal.

1. Introduction

Soybean is one of the fast emerging crops in the world and has also been described as the "wonder crop" because of its richness in protein (40%) and edible oil (20%). Amongst dietary proteins, soy protein is considered a complete protein as it contains all the essential amino acids along with several other nutritionally important micro and macronutrients. The biological value of soybean is thus considered to be roughly equivalent to that of animal protein (Velasquez & Bhathena, 2007). In addition, soy protein also contains compounds, e.g., anthocyanins, isoflavones, saponins, phospholipids (PLs), with a variety of biological properties that may potentially benefit human health (Kumari, Krishnan, Jolly, & Sachdev, 2014; Kumari et al., 2015).

A major factor that severely limits consumption of soy protein/soymeal is its offensive off-flavour, which has been attributed to its high content of polyunsaturated fatty acids (PUFAs) and the presence of lipoxygenase (LOX), which causes the peroxidation of those PUFAs containing *cis,cis*-1,4-pentadienes, such as linoleic acid and linolenic acid (Dahuja & Madaan, 2003). The products of this enzyme are subsequently acted upon by other enzymes leading to the production of off-flavour causing compounds, such as pentanal, hexanal, heptanal and nonanal, along with their unsaturated analogues (Dahuja & Madaan, 2004; Mandal, Dahuja, Kar, & Santha, 2014). These aldehydes are very reactive and bind covalently with proteins causing off-flavour problems both in soymeal and other soy products (Tewari, Kumari, Vinutha, Singh, & Dahuja, 2014).

Soymeal – a by-product of the soybean industry – has been found to contain significant levels of phospholipids, as these are not removed during oil extraction using conventional methods employing organic solvents such as hexane, carbon tetrachloride etc., because of their amphipathic nature. These residual phospholipids (PLs) and free fatty acids (FFAs) in soy protein isolate seem to be the starting precursors of off-flavours in soymeal. Even if carbonyl compounds are removed from soy protein isolate, PLs and FFAs in soy protein would continuously generate these carbonyls *via* autoxidation during storage (Arora & Damodaran, 2011a, 2011b). Furthermore, these PLs are tightly linked with oleosin proteins, raising the speculation about the role of oleosin proteins in off-flavour generation in soymeal.

Oleosin is the most abundant protein in the oil bodies of plant seeds, playing an important role in regulating oil body formation and lipid accumulation (Liu, Hua, & Qu, 2013). It has earlier been

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reported that the size of the oil bodies is correlated with oleosin content in seeds (Siloto et al., 2006; Tzen, Cao, Laurent, Ratnayake, & Huang, 1993), and therefore might be regulated by oleosins. Also, a positive correlation between oleosin levels and oil content has been shown in *Brassica napus* seeds (Hu et al., 2009). Furthermore, oleosins allow triacylglycerols (TAGs) to remain in discrete tightly packed organelles without coalescing. The small size of oil bodies provides a large surface area per unit TAG, which facilitates lipase binding and catalysis during seed germination (Huang, Chung, Lin, Hsing, & Huang, 2009). Since, oleosin proteins are tightly associated with PL molecules in soymeal, they might be expected to playing a role in off-flavour generation.

Biotic elicitors (having biological origin), such as jasmonic acid (JA), chitosan and salicylic acid (SA), have been reported to increase production of secondary metabolites, through activation of enzymes using various mechanisms. It has been reported by Kumari et al. (2014) that pre-harvest application of these biotic elicitors lead to reduction in protein oxidation in soybean seed by reducing the activity of off-flavour pathway enzymes and increasing the antioxidant activity of biomolecules. The purpose of the present study was to elucidate the roles of PLs and oleosins in off-flavour generation in soymeal and to devise methods for reducing the residual levels of PLs in soymeal to reduce off-flavour in soy products. Oil extraction using supercritical CO_2 and treatment of soybean seeds with biotic elicitors (chitosan and jasmonic acid) before oil extraction have been explored as the two strategies for this purpose.

2. Materials and methods

2.1. Reagents and standards

All the reagents, solvents and chemicals used in these experiments were analytical grade of high purity. Jasmonic acid and Chitosan were purchased from Sigma–Aldrich Co. (Steinheim, Germany). Lipoxygenase, Tween-20, methanol, hexane, ammonium persulfate (APS), 1-butanol and pyridine were purchased from Sisco Research Laboratory (Mumbai, India). Hydrochloric acid, perchloric acid ($HClO_4$), sodium hydroxide (NaOH), Tris–HCl buffer, magnesium chloride ($MgCl_2$), ethylene diaminetetraacetic acid (EDTA), dithiothreitol (DTT), sodium chloride, guanidine hydrochloride, bovine serum albumin (BSA), 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma–Aldrich Co. (Bangalore, India). All the chemicals for the purification of oil-body-associated protein by SDS–PAGE, *N,N′*-methylene bisacrylamide, Tris–HCl buffer, Tris buffer, sodium dodecyl sulphate (SDS), glycine, bromophenol blue, Coomassie Brilliant Blue R-250, glacial acetic acid, tetramethylethylenediamine (TEMED), phospholipase A_2 and dilinolylphosphatidyl choline were purchased from Sigma–Aldrich Co. (St Louis, MO). 1-Amino-2-naphthol-4-sulphonic acid (ANSA) and deoxycholate were obtained from Sigma–Aldrich Co. (Shanghai, China). Chloroform and ether were purchased from Amresco (India). Acetone, glycerol and analytical grade water were obtained from Himedia (India).

2.2. Seed materials

Four soybean seeds varying with respect to their off-flavour-generating potential were obtained. High off-flavour-generating varieties included SL-525 and Bragg, whilst PK 416 and EC 4172168 were taken as low off-flavour-generating genotypes. The seeds were powdered and defatted with two different oil extraction methods employing supercritical carbon dioxide ($SC\text{-}CO_2$) and organic solvent (hexane); phospholipid content and thiobarbituric acid (TBA) number were estimated in the soymeal obtained after oil extraction. Furthermore, seeds were also treated with biotic elicitors, *viz*., chitosan and jasmonic acid, for studying their effect on TBA number, protein profile and oleosin protein isolation from oil bodies, and PLA_2 activity of isolated oleosin proteins. For biotic elicitor treatments, soybean seeds were washed in distilled water, surface-sterilised with 70% ethanol, dried and treated with biotic elicitors (chitosan 4 and 8 mg/mL; jasmonic acid 3 and 5 μM) and finally kept in an incubator on germination paper for 8 days. Fresh elicitor solutions were added every day. After treatments, seeds were used for biochemical analyses.

2.3. Defatting of soybean seeds by cold percolation method and supercritical carbon dioxide ($SC\text{-}CO_2$)

2.3.1. Defatting of soybean seeds by cold percolation

Hexane was used as an organic solvent extraction medium in which sample was prepared and extracted according to Kartha and Sethi (1957). Extracted materials were dried, weighed and further used for phospholipids estimation.

2.3.2. Defatting of soybean seeds by supercritical carbon dioxide ($SC\text{-}CO_2$)

Fresh soybean seeds were cracked, dehulled and placed in an extractor (Waters MV-10 ASFE System). The extractor was sealed and brought to desired pressure whilst heating the vessel heater at 30 °C and electrical heat exchanger at 75 °C. With a CO_2 supply pressure of 150 bar, a flow rate of 45 g/min was maintained. After 45 min the extractor was depressurised, and the defatted materials were removed and used for phospholipids estimation (Nodar, Molero Gomez, & Martınez de la Ossa, 2002).

2.4. Estimation of residual phospholipids in soymeal

Phospholipids from soymeal were extracted by sonication with solvent mixture of $CHCl_3$:CH_3OH (1:2, v/v) (Arora & Damodaran). Estimation of phospholipids was done by using modified Bartlett (1959) method, in which organic phospholipid phosphorus was converted into inorganic phosphorus, which reacts with ammonium molybdate to form phosphomolybdic acid which on reduction and reaction with ANSA (1-amino-2-naphthol-4-sulphonic acid) forms a stable blue colour. Different aliquots (0.0, 0.2, 0.4, 0.6, 0.8 and 1 mL) of working standard (80 μg phosphorus/mL) and 1 mL aliquot of soymeal sample were pipetted out into a series of labelled test tubes. Concentrated perchloric acid (0.5 mL) was added to all the test tubes, including the test tubes labelled 'blank' and 'unknown', mixed and digested over a sand bath until they became colourless. Then the volume was made to 4.3 mL with distilled water. To this 0.5 mL of 2.5% ammonium molybdate solution were added. After 10 min 0.2 mL of ANSA (25 mg/mL) were added and the mixture was incubated at room temperature for 20 min. Absorbance was then taken at 660 nm against a blank. From the standard curve the concentration of phospholipid was calculated in the given sample. The colour produced is proportional to the concentration of phosphorus up to 1.5 μmoles in the reaction mixture. These values were expressed as the phospholipid (lecithin) content by multiplying phosphorus content by a factor of 25.

2.5. Estimation of thiobarbituric acid (TBA) Number

TBA number is a measure of the amount of malondialdehyde produced by the decomposition of various primary and secondary lipid peroxidation products. Its estimation is based on the reaction of TBA with malondialdehyde (MDA) to form a pink coloured MDA–TBA adduct (Ohkawa, Ohishin, & Vagi, 1979). Overnight, soaked defatted powder (0.2 g) was homogenised with 2.0 mL of distilled water and the homogenate was centrifuged at 15,000*g* for 30 min. The extract obtained was then filtered through filter