Effect of maleylation on physicochemical and functional properties of rapeseed protein isolate

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Abstract Influence of maleylation on the physicochemical and functional properties of rapeseed protein isolate was studied. Acylation increased whiteness value and dissociation of proteins, but reduced free sulfhydryl and disulfide content ($p < 0.05$). Intrinsic fluorescence emission and FTIR spectra revealed distinct perturbations in maleylated proteins' tertiary and secondary conformations. Increase in surface hydrophobicity, foaming capacity, emulsion stability, protein surface load at oil-water interface and decrease in surface tension at air-water interface, occurred till moderate level of modification. While maleylation impaired foam stability, protein solubility and emulsion capacity were markedly ameliorated ($p < 0.05$), which are concomitant with decreased droplet size distribution (d_{32}). *In-vitro* digestibility and cytotoxicity tests suggested no severe ill-effects of modified proteins, especially up to low degrees of maleylation. The study shows good potential for maleylated rapeseed proteins as functional food ingredient.

Keywords Maleic anhydride · Rapeseed protein · Emulsification · Cytotoxicity

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Introduction

The main bio-waste of rapeseed processing is the oilseed cake or press-cake, which has relatively high protein content (≈ 40 – 45 %), making it an attractive potential source of vegetable protein. Rapeseed proteins have been extensively studied in recent years, because of their film-forming and biodegradable character, and good techno-functional properties. A roadblock to the large-scale use of rapeseed press-cake protein is its unacceptable colour and most native proteins do not show functional properties desirable for food industries. The need for multiple functional food ingredients and fabricated foods has increased the pressure on food industries and researchers to develop different modification techniques to enhance and diversify the protein functionalities (Matemu et al. 2011). For the above-stated reason, efforts have been made to improve the functionalities of oilseed proteins, and probably, the best documented of these efforts involve chemical and enzymatic modifications. Numerous investigations through chemical modification (succinylation, acetylation, phosphorylation and sulfamidation) or enzymatic modification of rapeseed proteins have been undertaken (Gruener and Ismond 1997; Gerbanowski et al. 1999; Schwenke et al. 2000; Sánchez-Vioque et al. 2004); however, proteolysates from the latter method are frequently bitter and lacks satisfactory foam/emulsion stability (Franzen and Kinsella 1976). Moreover, there are indications that all peptides do not contribute equally to the reported biological activities and thus the purification of active peptides is needed (Aachary and Thiyan 2012), making it a cumbersome and expensive technique. Among the chemical modifications, acylation by succinylation or acetylation has been widely used to improve the functional properties of food proteins, where either succinic anhydride (negatively charged) or acetic anhydride (neutral) has been used, respectively. These anhydrides

interact with positively charged sites on the protein with the N-terminal amino group. This results in a change in the net charge of the protein and at sufficiently high level of modification, denaturation of protein occurs. Succinylation and acetylation have both been used to modify canola proteins with the degree of modification varying up to 62 % acetylation or succinylation (Gruener and Ismond 1997), 84 % succinylation (Paulson and Tung 1989) and 93 % succinylation (Gueguen et al. 1990). For canola, low levels of modification led to improved foaming and emulsifying activities due to improved interfacial absorption kinetics (Gueguen et al. 1990), but foam stability was adversely affected by acylation and emulsion stability decreased at higher levels of modification (Gruener and Ismond 1997). Heat-induced gel characteristics were also improved with the firmest gels being obtained at intermediate levels of succinylation (Paulson and Tung 1989). Additional advantages of these modifications are related with delayed Maillard reactions, but the resulting derivative could be less susceptible to proteolysis (Moure et al. 2006).

Modified canola proteins have unquestionably been shown to possess improved features over the native proteins. Despite this, there are very few reports in the literature that contain detailed analyses of such modified proteins and their application for targeted food formulations. Moreover, extensive literature is available on acetylation and succinylation of proteins and the characterisation of such products, however, reports on preparation and characterization of acylated protein derivatives using other dicarboxylic acid anhydrides apart from acetic and succinic anhydride are scarce. In this milieu, references on food protein modification using maleic anhydride (maleylation) are very limited; the only reported study was that of Lawal and Dawodu (2007), where the authors studied the functional properties of maleylated African locust bean protein. To date, maleylation of rapeseed proteins has not been reported. Understanding the structural changes in modified proteins is imperative for interpreting their changed surface properties. So, this paper examines the physicochemical and functional changes in rapeseed protein after progressive maleylation, while also taking into consideration the changes in cytotoxicity and in-vitro digestibility of this protein following modification.

Materials and methods

Rapeseed press-cake was obtained from Assam Khadi & Village Industries Board, Guwahati, India. Ground meal was defatted using diethyl ether for reducing the lipid content to <0.1 % (by Soxhlet method). Since the dark colour of oilseed protein is mainly caused by its phenolic compounds, as a result of the removal of them the colour of the product is expected to become lighter. So, defatted meal (10 g) was treated with 100 ml of methanol:acetone mixture (1:1 v/v) containing 0.2 % perchloric acid (Das Purkayastha et al. 2015). The

suspension was shaken at $215 \times g$ for 2 h (at $25 \pm 1^\circ\text{C}$) in an orbital shaker (Sartorius Stedim Biotech, CERTOMAT® IS) and then centrifuged (SIGMA 3-18 K Centrifuge) at $5366 \times g$ for 20 min (at 4°C). Treated meal was dried in a vacuum oven under reduced pressure (150 mmHg) at 40°C for 72 h.

Preparation of rapeseed protein isolate from treated meal (Das Purkayastha et al. 2015)

Meal extract was prepared by constant mixing of treated meal–solvent mixture (meal:solvent ratio = 1:30 (w/v)) in an orbital shaker set at $215 \times g$ (25°C) for 1 h. The solvent consisted of an alkaline solution of pH 11, to which sodium chloride (0.1 M) and sodium sulphite (0.4 %) were added. Subsequently, the slurry was centrifuged at $10,733 \times g$ for 20 min (at 4°C). The supernatant was filtered and the volume of clarified extract was noted. Ammonium sulphate was dissolved into the supernatant to 85 % saturation and the mixture was kept in ice bath for 3 h with gentle stirring and then centrifuged. The resulting precipitate was redispersed in water, adjusted to pH 7, dialyzed against Milli-Q water and finally freeze-dried to obtain rapeseed protein isolate. Protein purity of all the isolates was above 84 % (dry basis), based on Kjeldahl nitrogen ($\text{Nx}6.25$).

Maleylation of rapeseed protein

Acylation was carried out according to the protocol of Lawal and Dawodu (2007). Briefly, protein slurry (25 % w/v in distilled water) was adjusted to pH 9 using 1 N NaOH and cooled in an ice-bath. Maleic anhydride (MA) was added in small increments at the levels of 0.1, 0.2, 0.4, 0.6 and 0.8 g/g of protein, during which pH was maintained at 8.5–9.0 with constant stirring. The reaction was considered to be complete when the pH remained constant. Following exhaustive dialysis against water for 48 h at 4°C , the suspensions were freeze-dried to obtain maleylated proteins (coded as 0.1MA, 0.2MA, 0.4MA, 0.6MA and 0.8MA, respectively). Rapeseed protein without anhydride treatment was regarded as the control.

Extent of chemical modification

Trinitrobenzene sulphonic acid (TNBS) method for free amino groups estimation was used for determining modification level (Gruener and Ismond 1997). One millilitre each of 0.05 M Na_2HPO_4 and 0.1 % TNBS were added to 1 ml of protein solution (1 % w/v in 0.05 M NaCl, pH 9.2), which was then allowed to react at 60°C water-bath for 2 h. To stop the reaction, 1 ml of 10 % SDS was added, followed by 0.5 ml of 1 N HCl. Absorbance at 335 nm was read against reagent only blank (omitting protein). Absorbance of the control protein was set equal to 100 %. Degree of maleylation was calculated by the percent decrease in absorbance relative to that of the

control, because fewer amino groups were able to react with TNBS reagent.

Free sulphydryl group (SH) and disulfide bond (SS) were assayed by the method of Tang et al. (2009) and Deng et al. (2011). Protein samples (75 mg) were dissolved in 10 ml of Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, and 0.04 M EDTA, pH 8.0) containing 8 M urea. The solution was gently stirred overnight until a homogeneous dispersion was achieved. For SH content determination, 4 ml of the Tris-Gly buffer was added to 1 ml of protein solution. Then, 0.05 ml of Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid in Tris-Gly buffer, 4 mg/ml) was added, and absorbance was measured at 412 nm after 5 min. For total SH content [SH + reduced SS] analysis, 0.05 ml of β -mercaptoethanol (β -ME) and 4 ml of Tris-Gly buffer were added to 1 ml of the protein solution. The mixture was incubated for 1 h at room temperature. After an additional hour of incubation with 10 ml of 12 % trichloroacetic acid (TCA), the mixtures were centrifuged at 5000 g for 10 min. The precipitate was twice resuspended in 5 ml of 12 % TCA and centrifuged to remove β -ME. The precipitate was dissolved in 10 ml of Tris-Gly buffer. Then 0.04 ml of Ellman's reagent was added to 4 ml of this protein solution, and the absorbance was measured at 412 nm after 5 min. The calculations are as follows:

$$\mu\text{M SH/g protein} = (73.53 \times A_{412} \times D)/C \quad (1)$$

$$\mu\text{M SS/g protein} = (\text{SH}_{\text{total}} - \text{SH}_{\text{free}})/2 \quad (2)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration (mg/ml), D is the dilution factor, 5 and 10 are used for SH and total SH (SH + reduced SS) content analysis, respectively, and 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 is the molar absorptivity, and 10^6 is for the conversion from molar basis to $\mu\text{M/ml}$ basis and from mg solids to g solids.

Surface hydrophobicity (S_o) was measured by SDS binding method (Hiller and Lorenzen 2008) with slight modifications. Sodium dihydrogen phosphate dihydrate buffer (0.02 mol/L, pH 6.0) was prepared in demineralized water. Proteins were soluted in sodium dihydrogen phosphate buffer ($w/v = 1.0$ g/l). SDS reagent was prepared in sodium dihydrogen phosphate buffer ($w/v = 40.37$ mg/l). Methylene blue was dissolved in sodium dihydrogen phosphate buffer ($w/v = 24.0$ mg/l). The protein solution and SDS reagent were mixed ($v/v = 1/2$) and incubated for 30 min at 20 °C. Subsequently, the SDS-protein solution was dialyzed against sodium dihydrogen phosphate buffer ($v/v = 1/25$) for 24 h at 20 °C. Mixtures of 0.5 ml of dialysate, 2.5 ml of methylene blue, and 10.0 ml of chloroform were centrifuged at 2500 g for 5 min. The transmittance (%T) of the chloroform (lower) phase was assessed spectrophotometrically at a wavelength of $\lambda = 655$ nm. Chloroform served as solvent blank.

$$\text{Surface hydrophobicity } (\mu\text{g SDS}/500\mu\text{g protein}) = 62.305 \times \log[1/(\%T/100\%)] - 0.8349 \quad (3)$$

Surface tension measurement of aqueous protein solution (7 mg/ml) was conducted by applying the platinum ring method (6 cm diameter) with the aid of a tensiometer (Jencon, Sl. No. 315, Kolkata-28, India) after calibrating the instrument with water (72.0 mN/m) (Tsoukala et al. 2006).

Intrinsic fluorescence spectra were recorded in a Perkin Elmer LS55 fluorescence spectrophotometer (Perkin Elmer Corp., USA) using 2 mg/ml protein solution and background calibrated with Milli-Q water. The solution was excited at 290 nm to minimize the contribution of tyrosine residues.

Fourier transform infrared (FTIR) spectroscopy was carried out on solid samples by KBr pelleting method using Perkin Elmer Spectrum-100 model (Zhang et al. 2015). Secondary structure quantitations were performed by the Gaussian curve-fitting of the spectrum in the Amide I region (Shevkani et al. 2015).

Molecular weight (MW) determination by Gel Permeation Chromatography (GPC)

Lyophilized protein (4 mg) was dissolved in 0.5 ml of phosphate buffer (pH 11), vortexed and then mixed with 0.5 ml of

0.05 M NaCl, so that the final solution had a pH of 9.2 (Massoura et al. 1998). The solution was centrifuged for 15 min at $8586 \times g$ (4 °C), and the supernatant were filtered through a 0.22 μm cellulose membrane filter and analyzed by a Waters GPC system (515 HPLC Pump), equipped with a Waters HSPgel™ AQ 3.0 column (6.0 $\times$ 150 mm) and a Waters 2489 UV detector (Mao and Hua 2014). Following chromatographic conditions were applied: injection volume = 20 μl ; isocratic flow rate = 0.3 ml/min; elution solvent = Milli-Q water containing 0.3 M NaCl; and run time = 15 min. The UV absorbance was recorded at 280 nm and the spectra were processed using Empower 2 software (Waters, Milford, MA). A standard curve was obtained using a Gel Filtration Kit for protein MWs (MWGF70-1KT, SLBB8609, Sigma-Aldrich, USA), which contains aprotinin (6.5 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), and bovine serum (66 kDa).

In-vitro digestibility of protein was determined as described by Groninger and Miller (1979) after incorporating the modification from Zhong et al. (2012). Twenty milligram of pepsin was added to 700 mg of protein suspension having pH 1.5–2.0 (adjusted with 1 N HCl) and maintained at 37 °C in a water-

bath shaker. After 2 h, pH was raised to 7.0 with 1 N NaOH to stop the reaction. When peptic digestion was finished, the protein hydrolyzate was further digested by adding trypsin (20 mg trypsin to 700 mg of protein suspension, pH 7.0–8.0 borate buffer with 0.1 M CaCl₂). After 2 h of shaking (37 °C), the reaction was stopped by adding 20 % trichloroacetic acid, centrifuged at 4293 × g for 20 min. Nitrogen content released by the enzyme in the supernatant was directly measured by Kjeldahl method (Wanasundara and Shahidi 1997). Casein, a protein usually employed as standard in studies on human feeding, was used as reference (Salgado et al. 2012).

$$\% \text{ enzymic digestion} = \frac{\text{Nitrogen released by enzyme}}{\text{Total nitrogen of undigested protein}} \times 100\% \quad (4)$$

Colour characteristics were determined with Hunter Lab colorimeter (Ultrascan, VIS-Hunter Associates Lab., USA) and were expressed as L (lightness), a (redness/greenness), b (yellowness/blueness) colour units. The instrument (including 65°/0° geometry, D25 optical sensor, 10° observer, specular light) was calibrated with black and white tiles provided by the manufacturer. Whiteness was calculated as follows (Balange and Benjakul 2009):

$$\text{Degree of Whiteness} = 100 - \left[(100 - L)^2 + a^2 + b^2 \right]^{1/2} \quad (5)$$

Phenolic content of protein was determined according to our earlier report (Das Purkayastha et al. 2015) using JEOL 400 MHz ¹H-NMR system. Freeze-dried protein was extracted with 99.8 % methanol (1:20, w/v) in a round-bottom flask for 48 h with stirring. The extract was filtered through cotton wool, concentrated under vacuum, and an internal standard (N,N-Dimethyl formamide) was added and then re-suspended in deuterium oxide (1 ml). Singlet proton NMR peak at 3.25 ppm were identified as phenylpropanoid ester (sinapine or esterified sinapic acid).

Cytotoxicity assay

Human Embryonic Kidney (HEK-293 T) and Mouse embryonic fibroblasts (MEF) cells were grown in 24-well plates using complete culture media (10 % Fetal bovine serum (FBS) + 45 % high glucose Dulbecco's Modified Eagle's Medium (DMEM) + 45 % Nutrient F10 + 1 % antibiotic-antimycotic mix) at 37 °C with 5 % CO₂ and humidity. Trypan blue dye exclusion test was performed to check viability of cultured HEK cells upon treatments with the protein samples for 48 h. Proteins were solubilised in incomplete culture medium (DMEM and Nutrient F10 (1:1 w/w)). Different concentrations of protein samples (for HEK: 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/ml; for MEF: 1.0, 2.0, 3.0 and 4.0 mg/ml) were added to 40 % confluent cells and grown in a CO₂ incubator at

37 °C with 5 % CO₂ and humidity for 48 h. After the incubation period, cells were harvested using 0.25 % trypsin EDTA (Gybc Life technologies, UK) treatment followed by neutralization using complete culture medium. Cell suspensions were centrifuged at 200 g for 5 min at room temperature. Supernatant was discarded and the cell pellet was re-suspended in 100 µl of complete culture medium. Trypan blue (0.4 %) and cell suspension in the ratio of 1:1 (v/v) was prepared and incubated for 8 min, followed by counting of live and dead cells using a haemocytometer chamber in a phase contract bright field microscope. Percentage of viable cells was calculated as

$$\text{Cell survivality (\%)} = \frac{\text{Number of live cells}}{\text{Number of live cells} + \text{Number of dead cells}} \times 100 \quad (6)$$

Protein solubility (PS) was determined by dispersing 1 % (w/v) freeze-dried protein powder in distilled water (El-Adawy 2000). Suspension was agitated for 2 h at room temperature before centrifuging at 4293 × g for 20 min (4 °C). The formula used for calculating solubility is given in Eq. 7 (Li et al. 2015)

$$\% \text{ Solubility} = \frac{\text{Soluble protein content in supernatant}}{\text{Total protein in freeze-dried sample}} \times 100\% \quad (7)$$

Emulsion capacity (EC), emulsion stability (ES), foaming capacity (FC) and foam stability (FS) were studied by the methodology of Hassan et al. (2010), with slight modification. Oil-in-water (o/w) emulsions were prepared by blending soybean oil with aqueous protein solution (7 mg/ml) at a ratio of 3:7 (v/v). The mixture was sonicated in an ultrasonic water bath (Labotec Inc., UK) for 10 min to form emulsion.

Emulsion microstructure by fluorescent microscopy

Emulsion (1 ml) was stained with one drop of fluorescence dye Rhodamine B (0.025 % dissolved in water) and gently mixed by inversion. A 15 µl aliquot of the resulting emulsion was added to 1 ml of distilled water, stirred gently, and then a single drop was poured onto a glass-slide and observed via fluorescent microscope (LEICA DM 3000, Power: ebq 50 qc, USA) attached with a LEICA DFC 450C camera. The 568 nm laser line was used for excitation, inducing a fluorescent emission of Rhodamine B (Arancibia et al. 2015), detected between 600 and 700 nm (Vasbinder et al. 2003). Average droplet size (µm) was measured by ImageJ® software and represented by d_{32} (Karaca et al. 2011).

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (8)$$

where n_i is the number of droplets of diameter d_i .

Protein surface load (Γ_s), expressed as mg protein per m² fat surface area, was calculated by relating the adsorbed protein to the specific surface area of the emulsion (Cui et al. 2014; Tsoukala et al. 2006). Freshly prepared emulsions (as described earlier) were centrifuged for 30 min at $4293 \times g$ (4 °C) to accelerate creaming, and the lower serum (aqueous) phase (unadsorbed protein) was withdrawn carefully using a syringe. Protein concentration in the serum (C_{serum}) and that in the initial solution prior to emulsification (C_{initial}) was determined by Lowry method (Peterson 1977) using BSA as standard. Φ is the volume of oil phase used for emulsification (=0.3).

$$\Gamma_s = \frac{d_{32}(C_{\text{initial}} - C_{\text{serum}})(1 - \Phi)}{6\Phi} \quad (9)$$

Statistical analyses

All analyses were performed in triplicate and the mean value was calculated. Analysis of variance (ANOVA) and separation of means (by Tukey's HSD test) were performed by SPSS software (version 16.0, SPSS Inc., Chicago) and considered significantly different at $p < 0.05$.

Results and discussion

Extent of maleylation

Incremental addition of MA increased the extent of acylation from 29.3 % to 62.9 % of the ϵ -amino groups (Fig. 1a). These values were comparatively lower than those reported earlier on maleylated locust bean protein (Lawal and Dawodu 2007), which can be attributed to the differences in physicochemical nature of locust bean protein and rapeseed protein. Protein acylation reactions follow the carbonyl addition pathway (Fig. 1b) and the rate of reaction depends on the rate of nucleophilic attack: the π -electrons on the double bond of MA hinder the nucleophilic attack of the amino group (Lawal and Dawodu 2007). This may be one of the causes for lower level of modification in this study compared to those obtained by succinylation of canola 12S protein (Gruener and Ismond 1997). Even at the highest level of anhydride-to-protein ratio, not all free amino groups were maleylated. Such incomplete acylation is common for storage proteins of oilseeds (Wanasundara and Shahidi 1997), which may be due to spatial steric hindrance of some amino groups in protein.

Free sulfhydryl group (SH) and disulfide bond (SS)

The amount of free SH groups in the control were found to be 20.2 $\mu\text{M/g}$, which is comparatively close to the value reported for native rapeseed protein by He et al. (2014). In relation to the control, maleylation resulted in remarkable decreases in SH and

Fig. 1 a Effect of various concentration of maleic anhydride (MA) on degree of acylation of rapeseed protein (Points with different letters indicate significant difference ($p < 0.05$)); b the reaction scheme of MA with ϵ -amino group of protein

S-S content ($p < 0.05$) (Fig. 2a). This is because of the possible interaction of the acyl moiety with SH groups, as acylation is possible on all nucleophilic groups of amino acid residues, e.g. ϵ -amino groups of lysine, hydroxyl groups of serine and threonine, sulfhydryl group of cysteine, phenol group of tyrosine, and imidazole group of histidine (Franzen and Kinsella 1976). This observation corroborates with the findings of Groninger and Miller (1979) where one-third and one-fourth of the SH groups of fish proteins were found to react with succinic and acetic anhydrides, respectively. Apparently, the content of free SH in the maleylated samples under study presented a gradual rising trend with the increasing level of modification (Fig. 2a). This suggests either deacylation of SH in presence of high MA (Groninger and Miller 1979) or acylation-induced exposure of inaccessible thiol groups initially buried within the hydrophobic interior (mainly by protein unfolding and/or subsequent dissociation) has occurred. It should be pointed out that higher degree of unfolding and dissociation of protein molecules intensifies hydrophobic interactions, which in-turn favours the generation of S-S bonds (Condés et al. 2012). This might account for the overall marginal rise in S-S linkage as-well (Fig. 2a). Reduced availability of free thiol groups or the presence of negative maleyl groups in modified proteins probably gave rise to fewer disulfide bonds, as opposed to that of the control.

Fig. 2 a Free sulfhydryl and disulfide content; b surface hydrophobicity and surface tension of unmodified and modified proteins. Bars or points with different letters in each type of analysis indicate significant difference ($p < 0.05$)

Surface hydrophobicity (S_0)

Oilseed protein isolates are known for their high S_0 (Karaca et al. 2011). S_0 increased progressively up to 0.4MA, but decreased thereafter (Fig. 2b). This observation is similar to that of Matemu et al. (2011). The increased hydrophobic nature can be related to the gradual structural unfolding or exposure of initially buried hydrophobic clusters by progressive dissociation of protein molecules into its constituent subunits. Confirmation of this explanation is given by FTIR, GPC and intrinsic fluorescence of these samples (discussed later). Despite the presence of high amount of randomness and protein dissociation in 0.6MA and 0.8MA, the drop in hydrophobicity after exhaustive acylation may be due to the dense distribution of negatively charged maleyl residues, inhibiting Coomassie Blue dye (probe used for S_0 measurement) from approaching and binding to the exposed hydrophobic moieties on the protein surfaces (Strange et al. 1993). Similar behavior has been reported also for 1-anilino-8-naphthalenesulfonic acid (fluorescence probe for S_0 measurement) in modified soy protein hydrolyzates (Achouri and Zhang 2001) and acylated kidney bean protein (Yin et al. 2010).

Surface tension

Equilibrium surface tension of concentrated protein solutions are often around 45 mN/m (Lawal and Dawodu 2007), which is close to that obtained for the control at the air-water interface (~ 40 mN/m) (Fig. 2b). This value was however found to be much lower than that reported for BSA (57.7 mN/m) (Sánchez-Vioque et al. 2004), which points to the good adsorptive behavior and low surface tension of rapeseed protein at the air-water interface. Highly surface-active molecules were obtained by maleylation, the surface tension decreasing up to 36.9, 36 and 35.2 mN/m for 0.1MA, 0.2MA and 0.4MA, respectively (Fig. 2b). These values are close to those mentioned for acylated rapeseed peptides at air-water interface (Sánchez-Vioque et al. 2004). The values indicate that introduction of maleyl groups facilitated flexibility of the protein molecule by exposing previously buried hydrophobic portions to the interface, thus enhancing lowering of surface tension (Lawal and Dawodu 2007) and such samples are expected to show improved foamability (Jain et al. 2015). Samples with moderate level of maleylation were slightly more effective in reducing the interfacial tension compared to highly acylated ones (Fig. 2b). This can be credited to their respective S_0 ; nevertheless, the lower S_0 of 0.6MA and 0.8MA derivatives was probably offset by their high solubility, explaining their low surface tension values with respect to that of the control.

Intrinsic fluorescence spectra

λ_{max} of the fluorescence emission spectrum of the unmodified sample was determined to be 344.2 nm (Fig. 3), which is close to the reported value of 340 nm for 12S canola protein (Gruener and Ismond 1997). Except 0.8MA, maleylation

Fig. 3 Comparative representation of emission fluorescence spectra of different proteins (Excitation at 290 nm)

resulted in a red shift of $\lambda_{\max}$ as compared to the control (Fig. 3), whose extent varied depending on the level of anhydride used. Gruener and Ismond (1997) and Schwenke et al. (2000) observed similar shifts in fluorescence emission after succinylation and phosphorylation of canola/rapeseed protein, respectively. A red shift indicates the exposure of tryptophan (Trp) residues from the interior of protein molecule to the polar solvent/environment. This can be deduced from the conformational perturbations like partial unfolding, progressive dissociation or expansion of the native oligomeric structure (Matemu et al. 2011), a conclusion in accordance with the greater random coil feature and dissociated protein subunits as suggested by FTIR and GPC results, respectively. Some refolding mechanism or protein aggregation through hydrophobic interactions might have occurred in 0.8MA leading to less exposure of Trp groups to the polar solvent (Gerbanowski et al. 1999), as evident from the blue shift of its $\lambda_{\max}$ (Fig. 3). Maleylation also induced a strong quenching of the fluorescence, which proceeded with increasing extent of modification (Fig. 3). Based on the interpretation of Schwenke et al. (2000), decrease in fluorescence intensity was probably due to the attached maleyl groups and partly due to the aggregate formation at higher modification level. Kim and Kinsella (1986) reported that the decrease in fluorescence intensity of soy glycinin above 50 % succinylation indicated re-association of the unfolded polypeptides to occlude certain Trp residues.

Fourier transform infrared (FTIR) spectra

Visual inspection of the FTIR profile revealed subtle changes among the samples, as they had nearly identical infrared spectra (Fig. 4). The band observed at 1401 cm^{-1} (attributable to C=O bond of ionized carboxyl (COO^-) group) became more pronounced after modification with respect to the control. This is because acylation introduces additional carbonyl groups (Strange et al. 1993). With an increasing extent of maleylation, a sharp peak at $\sim 861\text{ cm}^{-1}$ and a shoulder at $\sim 1185\text{ cm}^{-1}$, implying the presence of CH bend of alkene (or ring substitution) and acyl C-O, respectively, became more prominent especially above 0.4MA (Fig. 4). Such signs provided obvious indications of the introduction of maleyl groups in the protein backbone. In addition to aromatic C-H bending, the peak at 861 cm^{-1} may also indicate the presence of an indole ring, possibly by the exposure of buried Trp or tyrosine residues (Zhao et al. 2004). Dissociated protein subunits in the acylated samples ($\geq 0.4\text{MA}$), as evident from their GPC chromatograms, most likely revealed the buried Trp residues. These results follow the trend observed in intrinsic fluorescence emission spectra and points to the unfolding and dissociation of proteins by high charge accumulation upon exhaustive acylation. Infrared spectral changes in Amide I region were used for the conformational analysis of the secondary

structure of proteins (Supplementary Fig. S1). Successive maleylation caused a drastic loss in α -helix content with a concomitant rise in β -structures and random coils (Table 1). These results are in good agreement with published data on modified 12S and 2S rapeseed proteins (Gerbanowski et al. 1999). Achouri and Zhang (2001) also found a pronounced tendency of soy protein hydrolysates towards randomness with increasing modification level. It is feasible to believe that under high electrostatic repulsion and steric hindrance of the bulky negatively-charged acyl groups, most α -helical chains might have unraveled, leading to random coils, and then some of these unraveled chains probably realigned into β -conformations (Byaruhanga et al. 2006), facilitated by hydrophobic interactions.

Gel Permeation Chromatograms

The control sample was mainly composed of 4 components, with estimated MW of 219, 71, 16 and 5.8 kDa, respectively (Fig. 5). These correspond well with the literature reports. Lower MW fractions (16 and 5.8 kDa) were the most abundant, possibly due to the reducing effect of sodium sulfite used in protein extraction protocol (cleaves disulfide linkages to form smaller polypeptides) (Li et al. 2012). High MW minor fraction (ranging from 219 to 239 kDa) probably represented cruciferin (He et al. 2014), whose amount reduced successively till 0.4MA and finally disappeared at 0.8MA. This is because cruciferin is highly susceptible to dissociation upon acylation (Gruener and Ismond 1997; Schwenke et al. 2000). Attachment of negatively-charged succinyl group or phosphorous oxychloride has been found to induce stepwise dissociation of oligomeric protein to its constituent subunits (Achouri and Zhang 2001; Schwenke et al. 2000). Analogous structural changes ought to be expected in case of MA treatment. At 0.1MA, the peaks having MW of 16, 5 and 3 kDa appeared (Fig. 5). The 5 kDa peak, major component of 0.1MA, was even more predominant at 0.2MA. The 16 and 5 kDa moieties decreased with progressive acylation in favour of higher and lower MW fractions. Furthermore, the decrease in 16 and 3 kDa peaks with acylation led to its complete diminution at 0.4MA along with the emergence of new peaks having MW of 13, 6 and 4 kDa. These observations suggest that repulsive forces between maleyl groups provoked the dissociation of protein and subsequently caused aggregation between unfolded proteins or dissociated low MW subunits via hydrophobic interactions and other weak bonds. Such dissociation followed by re-aggregation has been reported in a number of oilseed proteins. In addition, there is also a possibility that introduction of acyl groups may slightly expand the subunits, making them to change their hydrodynamic radii, so that their characteristic peaks would shift or appear at different retention time (Condés et al. 2012). Upon exhaustive acylation ($\geq 0.6\text{MA}$), the relative proportion of 6 and 3.8 kDa fractions were

Fig. 4 Fourier Transform Infrared spectra of unmodified and modified rapeseed proteins

distinctly increased at the expense of 13 kDa peak which finally disappeared at 0.8MA. Similar phenomenon was observed during succinylation of fababean proteins, where 11S oligomer formed 7S and 3S components and finally created only 3S monomer at the highest modification level (Krause et al. 1998). Thus, GPC results seem to reflect the formation of both products of dissociation and aggregation after maleylation.

In-vitro digestibility of protein

In-vitro digestibility of protein was determined using casein as a reference (100 % digestibility). The control presented a

reduced digestibility of 96.1 % compared to casein (Fig. 6a); the differences in conformational characteristics between the oilseed proteins and casein may largely account for this. Maleylation treatment at 0.1MA increased digestibility in comparison to the control ($p < 0.05$) (Fig. 6a), which may be due to subunits dissociation and subsequent unfolding of polypeptide chains, making them more accessible to enzyme attack (El-Adawy 2000). Further increase in acylation level reduced the digestibility; however, the differences were not significant in comparison to the control ($p > 0.05$), except 0.8MA. This is due to the inability of the enzymes to hydrolyze peptide bonds that involve derivatized or acylated amino groups (Groninger and Miller 1979), or the highly acylated proteins might have suffered structural rearrangement to form a more compact tertiary structure (as evidenced by fluorescence observations), making it hard for the enzymes to act on the requisite active sites for hydrolysis to occur. Overall the current results are partly in agreement with that of Achouri and Zhang (2001), and the values are high enough to assure that there is not any adverse effect of maleylation on digestibility of 0.1MA, 0.2MA, 0.4MA and 0.6MA.

Table 1 Estimated secondary protein conformations obtained in the Infrared (Amide I) spectral region of unmodified and modified rapeseed proteins

Proteins	Area (%)		
	α -helix	β -structure (β -sheet + β -turn)	Random coil
Control	27.33 $\pm$ 0.40 ^a	41.24 $\pm$ 0.65 ^a	31.43 $\pm$ 0.18 ^a
0.1 MA	21.20 $\pm$ 0.50 ^b	49.89 $\pm$ 0.97 ^b	28.91 $\pm$ 0.23 ^a
0.2 MA	20.16 $\pm$ 0.25 ^b	43.93 $\pm$ 0.41 ^{ac}	35.91 $\pm$ 0.68 ^b
0.4 MA	15.63 $\pm$ 0.30 ^c	42.79 $\pm$ 0.35 ^c	41.58 $\pm$ 0.62 ^c
0.6 MA	11.17 $\pm$ 0.41 ^d	57.38 $\pm$ 0.57 ^d	31.45 $\pm$ 0.37 ^a
0.8 MA	7.65 $\pm$ 0.42 ^e	54.79 $\pm$ 0.62 ^c	37.56 $\pm$ 0.55 ^b

Values are mean $\pm$ SD ($n = 2$). Means with the same superscript letter within one column were not statistically different ($p > 0.05$)

Degree of whiteness

Unmodified rapeseed protein displayed a light-brownish colour and gritty texture. Maleylation greatly lightened its colour to off-white or chalk-white, with exceedingly fluffy texture (Supplementary Fig. S2). It has been observed earlier that though rapeseed/canola protein portrays an off-white colour in dry powder state. Upon dissolution in water, however, their

Fig. 5 Gel Permeation Chromatograms of unmodified and modified proteins

solutions show brown colours of different intensities (Das Purkayastha et al. 2015; Xu and Diosady 2002); this brown colour is noticeable in the control. So following the previous protocol, colorimetric evaluation of protein was performed by scanning their aqueous solution (5 % w/v). The control had the lowest whiteness value (Fig. 6a), due to its brown colouration caused by residual polyphenols (~29.9 µg/g protein). All the acylated counterparts, having phenolic content ≤3 µg/g protein, had significantly higher whiteness values than the control ($p < 0.05$). The observation is congruent with the reports of Franzen and Kinsella (1976), and Wanasundara and Shahidi (1997). This dramatic improvement in colour can be explained with the blocking of the nucleophilic amino groups of protein with acyl groups, which can otherwise react with the electrophilic o-quinone (oxidation product of polyphenols) (Aewsiri et al. 2009). The pH condition (pH 8–9) used for acylation improves the reaction selectivity of acyl group towards ε-amino groups (most reactive due to its low pK_a and low steric hindrance) (El-Adawy 2000), and thus cause a significant decrease in the content of colour-forming quinones. There was

no significant difference in the whiteness of the maleylated products ($p > 0.05$).

Cytotoxicity assay

Compared to 0.6MA and 0.8MA, the physicochemical and functional properties (discussed later) of 0.1MA, 0.2MA and 0.4MA were found to be satisfactory. So, these samples were selected for cytotoxicity assay and compared with that of the control. Results from Trypan blue dye exclusion test indicated that the tested protein samples do not pose severe cytotoxicity to the HEK and MEF cells (Fig. 6b and c). Cell viability was found to be higher than 90 % and 80 % for HEK and MEF cell-lines, respectively, even in the presence of high protein concentration (3 mg/ml); however, further rise in protein level, especially the control and 0.4MA, these values were slightly reduced to 84 % and 68 % in HEK and MEF, respectively. Although plant polyphenols are usually regarded as valuable natural antioxidant, not all phenolics present in foods are beneficial and some may be of anti-nutritional concern. Factually,

Fig. 6 **a** *In-vitro* digestibility and Degree of Whiteness; Cytotoxic effect on **b** Human Embryonic Kidney (HEK-293 T) and **c** Mouse embryonic fibroblasts (MEF) cells. Bars or points with different letters in each type of analysis indicate significant difference ($p < 0.05$)

in a number of polyphenol-protein models, phenolics or their oxidized forms have been shown to actuate protein precipitation (Baxter et al. 1997; Charlton et al. 2002). So, in accordance with the notion that the presence of residual phenolic compounds in the control sample may trigger clumping of cells by interacting with available proteins on cell surfaces, as is also seen in hazelnut meal protein (Aydemir et al. 2014); dramatic morphological changes became obvious at ≥ 2 mg/ml, with prominent cell clustering (Supplementary Figs. S3 and S4). It is noteworthy to mention that the *in-vitro* cytotoxic/antiproliferative tests reported in the literature generally use very low concentration of the test material (in μ g/ml), whereas in the current study, isolates were tested at

much higher scale (in mg/ml). Therefore, a point is possibly reached as the concentration increases where unadsorbed protein molecules begin to accumulate. In case of 0.4MA, the reduction is presumed to be because of the high negative charge density of the molecule. Nevertheless, 0.1MA and 0.2MA showed appreciable cell viability with values as high as 92 % in HEK and ≈ 80 % in MEF, even at very high concentration (4 mg/ml). Cell viability results of this study are comparable or marginally better than those reported for micro- and nanoemulsions prepared with modified starch (Yu and Huang 2013).

Protein solubility (PS)

PS (at pH 7) was remarkably increased by maleylation (1.2–1.7 folds) (Fig. 7a). After acylation, the cationic amino groups on protein are converted to net negative charge, which causes repulsion among the maleyl carboxyl and the native carboxyl groups. Resulting electrostatic repulsion promotes protein unfolding and penetration of water molecules is physically easier because of the expanded/loosened state of the polypeptides (Franzen and Kinsella 1976). This result lends credence to similar observation by Lawal and Dawodu (2007), and Gruener and Ismond (1997). Pronounced improvement in PS of 0.6MA and 0.8MA is mainly attributed to increased protein dissociation into low MW subunits, which results in better protein-water interactions (Lawal 2005).

Emulsion properties

Increases in EC observed following maleylation (1.3–1.8 folds) may be a reflection of the increased solubility and molecular flexibility due to unfolding and consequent exposition of the reactive hydrophobic clusters on protein surface (Fig. 7b). These developments facilitate re-arrangement of the lipophilic and hydrophilic residues of protein at the interface, improving EC. Moreover, decrease in size due to partial dissociation may enable the protein to diffuse/migrate more quickly to the oil-water interface; especially causing enhancement in EC of highly acylated derivatives (Fig. 7b), despite their low S_0 . These observations are in line with the earlier reports (Lawal and Dawodu 2007). Likewise, ES of acylated derivatives increased over the control till 0.2MA (1.5-fold), after which it reduced gradually (Fig. 7b). Improvement in ES at low levels of maleylation can be due to the low net charge of the acylated protein layer formed around oil droplets, which increases electrical potential (of the ionized interfacial film) and imparts coalescence stability (Lawal 2005). On the contrary, decrease in ES above 0.2MA can be due to low MW subunits of such acylated counterparts, which may be insufficient to form thicker interfacial films resulting in less stable emulsion (Matemu et al. 2001). It is noteworthy that initial increase in PS by acylation facilitated enhanced interaction

Fig. 7 **a** Solubility (at pH 7) of the unmodified and modified rapeseed proteins; **b** Impact of different proteins on Emulsion Capacity (EC) and Emulsion Stability (ES); **c** Foaming Capacity (FC) and Foam Stability (FS). Bars or points with different letters in each type of analysis indicate significant difference ($p < 0.05$)

between oil and aqueous phases; however, as the solubility is increased, probably a point was reached where further increase in solubility (by modification) led to accumulation of proteins in the aqueous phase instead of being at the interface, thereby deteriorating ES. Similarly, in case of casein-stabilised emulsions, the presence of excess protein in the continuous aqueous phase (due to high solubility) proved deleterious, since the micelles induce depletion flocculation (Dickinson and Golding 1997). It can also be assumed that high negative charge density maintains the molecule in a highly unfolded state and inhibits re-arrangement processes at the droplet surface by repulsive effect (Schwenke et al. 1998).

Foaming properties

Unlike emulsification, FC was nearly unaffected or slightly increased till 0.4MA ($p > 0.05$), and then decreased up to 3.4-folds at the highest level of modification ($p < 0.05$) (Fig. 7c). The pattern here is congruent to the observations on surface tension (Fig. 2b). The same structural alterations of the protein molecules that are important for emulsification are also believed to be responsible for foamability; however dispersion interactions between proteins and oil-water interfaces are always attractive whereas those between proteins and air-water interfaces are generally repulsive, and because of this difference proteins are adsorbed much more readily at the oil-water interface than at the air-water interface (Damodaran 2005). Thus foamability of a protein may not be analogous to its emulsion behavior. In highly acylated proteins, excessive repulsion due to high charge density maintains the molecule in a highly unfolded state and inhibits re-arrangement process at the interface, leading to deterioration in surface functionality (Schwenke et al. 1998). Similarly, the decrease in FS (Fig. 7c) following modification is reasonably due to repulsive effect of maleyl groups that limited protein-protein interactions, hindering the formation of a continuous network around air bubbles (Lawal and Dawodu 2007). Similar behaviour has been reported for acylated mung bean isolates (El-Adawy 2000). Therefore, maleylated rapeseed protein may not be suitable in food systems that require high FS such as cake and ice cream.

Emulsion microstructure by fluorescent microscopy

Maleylated proteins afforded smaller emulsion droplets when compared to unmodified one; the decrease in d_{32} (Fig. 8a) increases interfacial area and hence the EC. This is a reflection of increased solubility and looser structure of modified proteins. So, if the molecules anchor at the interface more quickly, smaller average droplet sizes will result (Sánchez-Vioque et al. 2004). It is feasible to believe that the electrostatic repulsion between droplets, due to negative charge of maleylated proteins, is expected to prevent inter-droplet protein-protein interaction, therefore preventing flocculation. This is evident from the typical smooth surface of the microspheres fabricated by most of the samples under study (Fig. 8b). In sharp contrast, emulsion droplets stabilized by 0.6MA and 0.8MA showed rugged surfaces (Fig. 8b). The latter observation is because of the creation of numerous small droplets which appeared to be highly flocculated. This may indicate that such acylated samples contained some low molecular weight fractions that were very surface active, enabling the formation of small droplets, but the content of emulsifier (protein) present at the newly created oil-water interface was not sufficient for full coverage of droplet surface (Chen et al. 2011), therefore, bridging flocculation of droplets became possible. This in-turn

Fig. 8 **a** Average droplet-size (d_{32}) and protein surface load (Γ_s) of o/w emulsions stored at 25 °C for $t = 0$ h (Bars or points with different letters in each type of analysis indicate significant difference ($p < 0.05$)); and **b**

Fluorescence micrographs of the o/w emulsion stabilized by (i) control, (ii) 0.1MA, (iii) 0.2MA, (iv) 0.4MA, (v) 0.6MA, and (vi) 0.8MA stored at 25 °C for $t = 0$ h (Scale bar represents 100 μm)

is caused by low S_0 and excessive charge density, preventing intra-molecular protein–protein interaction. Poor ES of such samples can be anticipated from their flocculated droplets. As Rhodamine-B stains the protein component, individual droplets encapsulated by protein and/or regions rich in protein appear as red patches, which seems to be much less in Fig. 8b(v) and b(vi) compared to other samples. This observation also helps in drawing the inference that proteins from 0.6MA and 0.8MA are less available at the interface.

Protein surface load (Γ_s)

Effectiveness of a protein as an emulsifier also depends strongly on the protein surface load at saturation (Γ_s). Γ_s for most food proteins have been reported in the range of 1–10 mg/m^2 (Chen et al. 2011). Our result is comparable with that of legume protein (Tsoukala et al. 2006) and suggests that maleylation increased Γ_s till 0.4MA and then lessened it with further modification (Fig. 8a). A possible explanation could be

sought in light of much higher flexibility of the modified protein molecules, compared with the native one which enables them to adsorb at a much higher rate at the newly formed interfaces during emulsification. Native proteins seem to possess a much more globular and rigid structure and thus have a slow conformational change at the interface (Tsoukala et al. 2006). High Γ_s may also point to the formation of protein layers around fat globules that renders the emulsion more stable and resistant to coalescence. If the molecules are highly charged, it becomes increasingly difficult for more molecules to adsorb to the surface because of the electrical potential established by previously adsorbed molecules, creating an energy barrier to further adsorption (Tsoukala et al. 2006). This might be the possible cause for the reduction of Γ_s in 0.6MA and 0.8MA (Fig. 8a). Although highly acylated samples adsorbed at a lower degree, their reduced molecular sizes afforded rapid mobility/spreading, leading to the creation of a film around oil droplets faster (Tsoukala et al. 2006), hence higher EC. In the same way, protein hydrolyzates or peptides

often increases emulsifying activity index and produces smaller oil droplets than the intact protein, but produces very unstable emulsions and foams (Damodaran 2005).

Conclusion

Maleylation appeared to be effective in enhancing several surface functionalities of rapeseed protein. Improved whiteness, solubility, together with good digestibility and biocompatibility, of maleylated proteins allow reappraising the value of underutilized oilseed-cakes as a source of proteins useful for the food industry. There are good relationships between physicochemical and functional properties of acylated proteins. A detailed evaluation of the digestibility and nutritional quality of maleylated proteins require more elaborate *in-vivo* tests to support these findings.

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