



Cocoa protein crosslinking using Maillard chemistry

R. Jumnonpon^a, S. Chaiseri^a, P. Hongsrabhas^a, J.P. Healy^b, S.J. Meade^b, J.A. Gerrard^{b,c,*}

^a Department of Food Science and Technology, Kasetsart University, Bangkok, Thailand

^b Biomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, Christchurch, New Zealand

^c Riddet Institute, Palmerston North, New Zealand

ARTICLE INFO

Article history:

Received 18 October 2011

Received in revised form 3 January 2012

Accepted 28 February 2012

Available online 7 March 2012

Keywords:

Cocoa

Protein–protein crosslinking

Glutaraldehyde

Methylglyoxal

Polyphenol

ABSTRACT

Protein crosslinking *via* the Maillard reaction has been shown to alter the functional properties of several food proteins, but the potential to use this chemistry to alter the functional performance of cocoa proteins has yet to be fully explored. This study investigated the potential of employing cocoa protein crosslinking during processing. Cocoa protein solutions were extracted from dried cocoa beans and the resulting cocoa protein solutions were treated with 2–300 mM glutaraldehyde and methylglyoxal, which established model compounds for Maillard crosslinking chemistry. SDS–PAGE analysis showed remarkably few cross-links of the cocoa protein solutions, although lysine and particularly arginine availability were both reduced during the incubations. These results suggest that cocoa proteins are not greatly susceptible to intermolecular cross-linking *via* Maillard chemistry, perhaps due to non-cross-linking modifications to the arginine residues, and/or to interference from high levels of polyphenols in the cocoa bean.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Proteins are the second most abundant constituent of cocoa beans, which are comprised of lipid (62.9%), protein (17.5%), fibre (5.9%), water (5%), theobromine (2.9%), calcium (29.2 mg/100 g) and phosphorous (201 mg/100 g) (Aremu, Agiang, & Ayatse, 1995). Cocoa proteins include albumins (water-soluble), globulins (salt-soluble), prolamins (alcohol-soluble) and glutelins (soluble in dilute acids or alkali) (Zak & Keeney, 1976). The proteins of cocoa beans have been described as 52% albumin and 43% globulins (Voigt, Biehl, & Wazir, 1993). Changes in the protein composition of cocoa beans have been noted during fermentation and roasting (de Brito et al., 2000). During fermentation, total measurable cocoa proteins, along with the albumin fraction, have been shown to increase with time, while the globulin fraction decreased (Amin, Jinap, & Jamilah, 1997; Voigt et al., 1993). Moreover, peptide–nitrogen and free amino acids increased with fermentation time, while total protein concentration decreased (Adeyeye, Akinyeye, Ogunlade, Olaofe, & Boluwade, 2010; de Brito et al., 2000; Hashim, Selamat, Muhammad, & Ali, 1998). During roasting, the albumin fraction was shown to decrease from 35.65% to 18.10% (Abecia-Soria, Pezoa-Garcia, & Amaya-Farfán, 2005); total protein and free amino acids also decrease with roasting time (Abecia-Soria et al., 2005; de Brito et al., 2000).

Roasting of cocoa beans generates cocoa aroma and flavour *via* the Maillard reaction. During roasting, the reactive carbonyl groups of sugars interact with the nucleophilic amino groups of amino acid residues (Granvogl, Bugan, & Schieberle, 2006). Through the Maillard reaction, all of the cocoa aroma precursors interact to produce cocoa flavour components, such as alcohols, carboxylic acids, aldehydes, ketones, esters, pyrazines and amines. Cocoa specific aroma has been described in terms of sweet, nutty, caramel and chocolate-like notes associated with trimethylpyrazine, tetramethylpyrazine, 2,3-butanediol, dodecanoic acid, phenylethyl alcohol, ethanone, benzeneacetaldehyde and 1,4-bis(morpholinoacetyl) piperazine (Misnawi & Ariza, 2011).

The Maillard reaction of proteins does not only result in the generation of flavours and colours, but also changes the texture of foods if protein cross-linking occurs (Gerrard, 2002). Protein–protein cross-links have been shown to influence the gelation of globular proteins such as egg white, bovine serum albumin and soy protein isolate (Kaye, Easa, & Ismail, 2001; Mitchell & Hill, 1995; Sun, Hayakawa, & Izumori, 2004). When covalent bonds are formed between these proteins *via* the Maillard reaction, the solubility of the protein is reduced, leading to changes in the rheological properties and the water holding capacity of the gel (Kaye et al., 2001; Mitchell & Hill, 1995; Sun et al., 2004). Gluten proteins are also susceptible to protein–protein cross-linking, which reduced loaf size and improved the crumb strength of bread (Gerrard, Brown, & Fayle, 2003a).

Model studies of the Maillard crosslinking of food proteins have employed efficient cross-linkers, such as methylglyoxal and glutaraldehyde to mimic the impact of *in situ* Maillard crosslinking

* Corresponding author at: Biomolecular Interaction Centre and School of Biological Sciences, University of Canterbury, Christchurch, New Zealand. Tel.: +64 33667001; fax: +64 33642590.

E-mail address: juliet.gerrard@canterbury.ac.nz (J.A. Gerrard).

(Gerrard, Brown, & Fayle, 2002, 2003b; Meade, Miller, & Gerrard, 2003). This work aimed to extend this work to the proteins of cocoa by using glutaraldehyde and methylglyoxal as crosslinkers to investigate the potential of crosslinking on the functionality of cocoa proteins.

2. Materials and methods

2.1. Sample preparation

Dried, non-roasted cocoa beans (*Theobroma cacao*) used in this experiment were of the Forastero variety and obtained from a local farm in the South of Thailand. Dried cocoa beans were cracked to remove shells and germs. The non-roasted cocoa nibs were finely ground to produce cocoa mass using a rotor mill at 12,000 rpm, at room temperature. The cocoa mass was then defatted by Soxhlet apparatus for 24 h using hexane. Cocoa proteins were extracted from defatted cocoa mass using either 0.5% SDS–0.05 M phosphate buffer, pH 6.9 or 1% (v/v) 2-mercaptoethanol–8 M urea. Cocoa samples (0.1 g) were weighed into 1.5 ml Eppendorf tubes and combined with 1 ml of the extraction reagent. The samples were mixed every 5 min for 1 h using a vortex mixer. After centrifugation at 3000g for 10 min the clarified supernatants were pooled. The precipitates were further extracted with 0.5 ml of extraction buffer. The clarified supernatants were pooled and stored at -18°C (Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007). All extractions were carried out in duplicate.

2.2. In vitro incubations

Cocoa protein solutions (180 μl) were pipetted into Eppendorf tubes and combined with 20 μl of glutaraldehyde or methylglyoxal (Sigma–Aldrich, MO, USA) stock solutions, which varied according to the desired final concentration (2, 5, 10, 20, 50, 100, 200 and 300 mM). The final volume was 200 μl . Incubations were carried out at room temperature (20–25 $^{\circ}\text{C}$) for 2 h. All incubations were carried out in duplicate.

2.3. SDS–PAGE analysis

Protein–protein crosslinking was monitored by in-house electrophoretic methods (Fayle et al., 2001). All materials were obtained from Invitrogen Corporation (CA, USA). The NuPAGE LDS sample buffer (5 μl) was added to 20 μl of the incubated samples and the mixtures were heated at 70 $^{\circ}\text{C}$ for 10 min before centrifugation with a Spectrafuge 16 M Mini Centrifuge (Labnet, NJ, USA) at 5000 rpm for 5 min. The samples (10 μl for extractions in 0.5% SDS–0.05 M phosphate buffer or 3 μl for extractions in 1% (v/v) 2-mercaptoethanol–8 M urea) were loaded on NuPAGE 4–12% Bis–Tris gels and the gels run at a constant 200 V for 50 min. All samples were analysed in duplicate.

2.4. Determination of lysine residues

Lysine availability was monitored by a modification of the o-phthalaldehyde (OPA) method as previously described (Gerrard et al., 2003b). The OPA (Sigma–Aldrich, MO, USA) reagent was prepared by mixing 25 ml bicine (1.6% (w/v), pH 9.4) with 40 mg of OPA dissolved in 1 ml methanol and 100 μl of 2-mercaptoethanol. The final volume of OPA reagent was adjusted to 50 ml with 1-propanol. The standard curve of fluorescence intensity was prepared using 0–4 mM of *N*- α -acetyl-L-lysine. The incubated samples (50 μl) were added to Eppendorf tubes containing 950 μl of OPA reagent and mixed by vortexing, then incubated for 2 min at room temperature before their fluorescence intensity was

measured using a FLUOstar OPTIMA – fluorescence plate reader (BMG LABTECH, Ortenberg, Germany) at excitation/emission wavelengths of 320/485–12 nm (gain at 1000 units) in a Greiner microplate. The concentration of lysine residues in the samples was quantified by comparison of the fluorescence intensity to the values from the standard curve. The measurement was carried out six times for each sample.

2.5. Determination of arginine residue

Arginine residues were estimated by a fluorometric method using 9,10-phenanthrenequinone (Schmitt, Schmitt, Münch, & Gasic-Milencovic, 2005). The incubated samples (100 μl) were added to 300 μl of 9,10-phenanthrenequinone (Sigma–Aldrich, MO, USA) (670 μM in absolute ethanol) before 50 μl of 2 M sodium hydroxide was added. The mixtures were vortexed and incubated at 60 $^{\circ}\text{C}$ for 3 h. The samples were vortexed every 30 min during the incubation. Subsequently, 450 μl of 2 M hydrochloric acid was added to the mixture, which was vortexed. The samples (200 μl) were loaded into microplates and the fluorescence intensity measured at excitation/emission wavelengths of 320/390–10 nm (gain set at 1000 units) using a FLUOstar OPTIMA – fluorescence plate reader (BMG LABTECH, Ortenberg, Germany). The concentration of arginine residues in the samples was quantified by comparison to a standard curve obtained from 0–0.12 mM *N*- α -acetyl-L-arginine. The determination was repeated six times for each sample.

2.6. Protein quantification

Quantification of the protein content of defatted cocoa was performed by measuring total nitrogen by the Kjeldahl method (AOAC 970.22, 2000). Protein nitrogen was converted to mg protein using the coefficient 6.25. The measurement was performed in triplicate.

2.7. Determination of protein in the extracts

The protein content of extracted cocoa protein solutions was assessed using the Bradford method (Bradford, 1976). Bradford reagent was obtained from BioRad (NSW, Australia). A standard curve plotting absorbance versus concentration was obtained using bovine serum albumin in the concentration range 10–100 $\mu\text{g}/\text{ml}$. The incubated samples (20 μl) were added to Eppendorf tubes containing 780 μl of distilled water, then 200 μl of Bio–Rad Bradford reagent was added and the samples were briefly vortexed. The mixture was left at room temperature for 6 min and subsequently the absorbance was read at 595 nm. The concentration of the extracted protein solutions were quantified from the standard curve. The measurement was repeated six times.

2.8. Total polyphenol content

The total phenols were determined using the Prussian Blue assay (de Brito, García, & Amâncio, 2002). The sample (50 μl) was added to 25 ml of distilled water, followed by the addition of 1.5 ml of 0.1 M $\text{FeNH}_4(\text{SO}_4)_2$ in 0.1 M HCl. After exactly 20 min, 0.008 M $\text{K}_3\text{Fe}(\text{CN})_6$ 1.5 ml was added. The absorbance was read after 20 min at 720 nm. A standard curve was constructed using tannic acid. The measurement was carried out six times.

2.9. Statistical analysis

Significance differences between means were determined by analysis of variance (ANOVA), using the SPSS statistical programme. The level of significance used was 95%.

3. Results and discussion

3.1. Extraction of cocoa proteins

The protein content of defatted cocoa beans was determined by the Kjeldahl method to be 213.7 mg proteins/g (Table 1), in general agreement with published data for unroasted cocoa beans (de Brito et al., 2000). Cocoa proteins were extracted using two different extraction buffers, following the methods of Yasir et al. (2007) and the protein content in the extracted samples was analysed by the Bradford method. As shown in Table 1, the two extraction buffers led to different yields of solubilised proteins. Using buffer containing 2-mercaptoethanol and 8 M urea enabled extraction of approximately 4.5-fold more cocoa proteins, presumably due to the reduction of disulphide bonds and protein unfolding. The proteins extracted with the SDS buffer (Section 2.1) yielded only 15.6 mg/g defatted cocoa particles, suggesting that most of the cocoa proteins remained present as insoluble complexes. Consistent with this, cocoa proteins extracted with 1% (v/v) 2-mercaptoethanol-8 M urea showed a higher content of 70.1 mg/g. Extraction of protein from defatted cocoa particles with the SDS- and urea-containing buffers (Section 2.1) left unextractable proteins, approximately 92.7% and 67.2% of proteins, respectively. This result suggests that the cocoa proteins might form strong complexes with other compounds in the cocoa bean, such as the insoluble protein/tannin complexes from oxidative polymerisation of polyphenols during cocoa fermentation (Redgwell, Trovato, & Curti,

2003). Moreover, glycoproteins have been reported in the cytoplasm of cocoa seed (de Brito et al., 2000).

SDS-PAGE analysis of the two extracts showed that they contained overlapping profiles of individual proteins (compare the second lane in Figs. 1a and 2a to lane 2 of Figs. 1b and 2b). Proteins extracted from defatted cocoa mass with SDS-containing buffer gave four major bands, 47, 21, 14.5 and below 10 kDa, on the SDS-PAGE (Fig. 1a, lane 2; Fig. 2a, lane 2). The polypeptides with the molecular weights of 14.5 and 47 kDa were consistent with previously reported molecular weights for the globulin proteins (Amin et al., 1997; Voigt et al., 1993) and the most prominent protein, with the molecular weight of 21 kDa, was likely to be albumin, as previously reported in the literature (Amin et al., 1997; Buyukpamukcu et al., 2001; Lerceteau, Rogers, Pétiard, & Crouzilat, 1999). The polypeptide with a molecular weight below 10 kDa was possibly a breakdown product generated during fermentation (Buyukpamukcu et al., 2001).

The sample extracted with urea (Fig. 1b, lane 2; Fig. 2b, lane 2) had an additional two protein species, one in the region between 80 and 110 kDa, and another at 31 kDa. The 31 kDa polypeptide was possibly the globulin previously reported by Kratzer et al. (2009) and Lerceteau et al. (1999).

3.2. Incubation of extracted cocoa proteins with glutaraldehyde and methyl glyoxal

Following previous methods (Yasir et al., 2007), both protein extracts were treated with various concentrations of glutaraldehyde and methylglyoxal at room temperature for 2 h. SDS-PAGE analysis of cocoa proteins extracted with SDS-containing buffer and incubated with glutaraldehyde are shown in Fig. 1a. The extracted proteins were not seen to crosslink until the concentration of glutaraldehyde reached over 10 mM, at which point the loss of individual protein bands was accompanied by a smearing of bands, particularly at high glutaraldehyde concentrations. Again, the SDS-PAGE profiles of cocoa proteins extracted with urea and incubated with different concentrations of glutaraldehyde are presented in Fig. 1b. In this case, little crosslinking is seen. Fig. 1b shows slight loss of protein in the 31, 47 and 80–110 kDa proteins when concentrations of glutaraldehyde were increased, but the proteins at 21, 14.5 kDa and between 3.5 and 10 kDa appear resistant to

Table 1
Protein contents of defatted non-roasted cocoa particles.

Samples	Protein content (mg/g)	% Protein yield ^C
Defatted non-roasted cocoa particles ^A	213.7 ^c	–
Proteins extracted with 0.5% SDS-0.05 M phosphate buffer, pH 6.9 ^B	15.6 ^a	7.3
Proteins extracted with 1%(v/v) 2-mercaptoethanol-8 M urea ^B	70.1 ^b	32.8

^{a,b,c} Significant differences amongst means ($P \leq 0.05$) $n = 6$.

^A From Kjeldahl analysis.

^B From Bradford analysis.

^C As percent of total protein in defatted non-roasted cocoa particles.

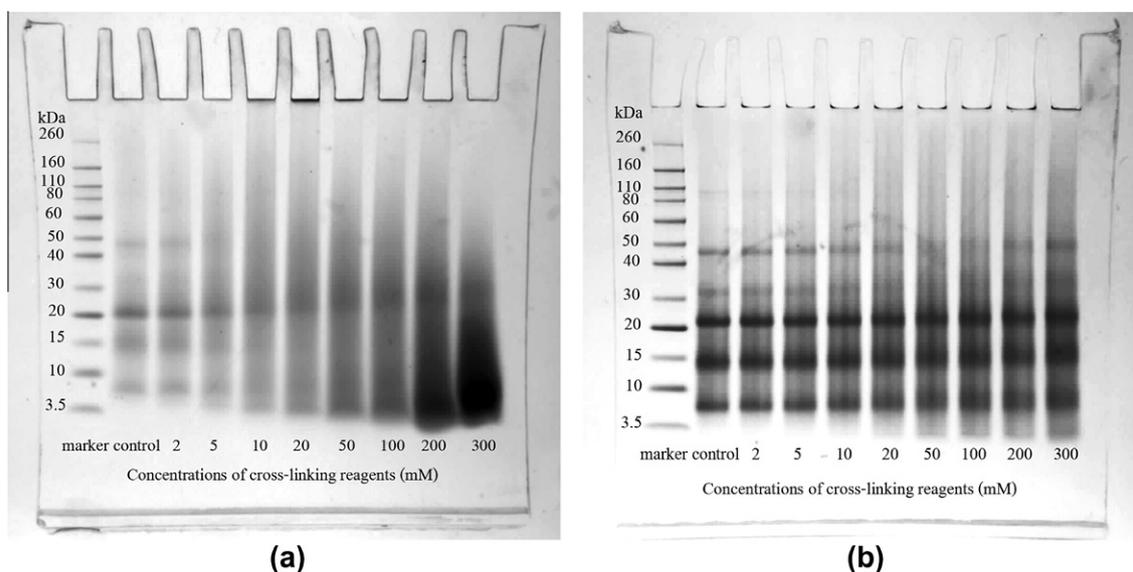


Fig. 1. SDS-PAGE profile of cocoa protein incubated with 2, 5, 10, 20, 50, 100, 200 and 300 mM glutaraldehyde, extracted with SDS-containing buffer (a) and extracted urea (b).

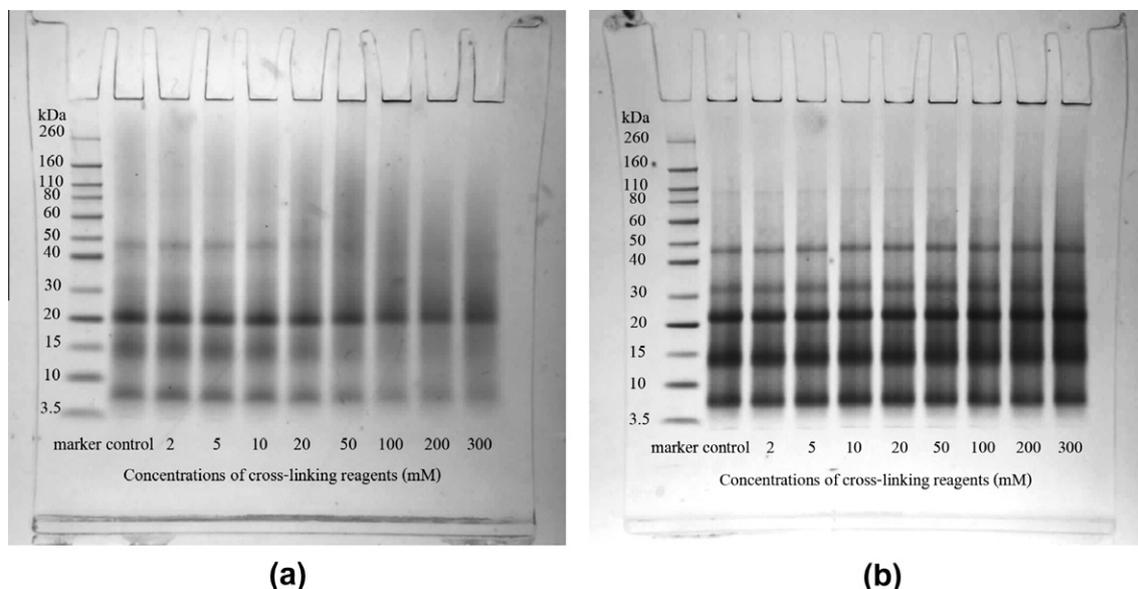


Fig. 2. SDS–PAGE profile of cocoa protein incubated with 2, 5, 10, 20, 50, 100, 200 and 300 mM methylglyoxal, extracted with SDS-containing buffer (a) and extracted with urea (b).

intermolecular crosslinking. These data indicated that only a few proteins, particularly those with high molecular weights, were cross-linked by glutaraldehyde.

This result is in contrast to results reported on other food proteins crosslinked with glutaraldehyde. Gelatin was reported to be crosslinked by 0.05% glutaraldehyde, with the extent of crosslinking increasing with glutaraldehyde concentration up to a maximum value of about 98% at 1% wt glutaraldehyde (Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001). The proteins bovine albumin and casein, crosslinked with glutaraldehyde (volume fraction 0.25%), increased their molecular mass by 20- and 40-fold, respectively (Silva, Sousa, Gübitz, & Cavaco-Paulo, 2003). Gerrard et al. (2003a) reported that wheat protein fractions (albumins and globulins, gliadins, SDS-soluble glutenins and SDS-insoluble glutenins) incubated with 10 mM glutaraldehyde formed intermolecular crosslinks immediately upon addition of glutaraldehyde. In legume lectins, 1% glutaraldehyde can induce cross-linking (Chatterjee & Mandal, 2003) and Yasir et al. (2007) found that soy proteins quickly formed intermolecular crosslinks with incubation of 1 mM glutaraldehyde at 30 °C.

The SDS–PAGE results of cocoa proteins extracted with 0.5% SDS–0.05 M phosphate buffer, pH 6.9 and treated with methylglyoxal did not show any crosslinking until the concentration of methylglyoxal reached 50 mM (Fig. 2a). The 47, 21, 14.5 kDa and smaller protein bands were lost, with a concomitant increase in smeared higher molecular weight material (Fig. 2a). The SDS–PAGE profiles of cocoa proteins extracted with 1% (v/v) 2-mercaptoethanol–8 M urea and incubated with different concentrations of methylglyoxal observed a similar effect to that observed with glutaraldehyde (Fig. 2b). The results suggest that the intermolecular cross-linking of cocoa proteins via the Maillard reaction occurred to only a minor extent in the extracts. In contrast, other proteins can be more effectively cross-linked with methylglyoxal. Bovine serum albumin showed crosslinking with 10 mM methylglyoxal at 37 °C after incubating for 2 h (Uchida et al., 1997). RNase A from bovine pancreas formed crosslinks immediately upon addition of methylglyoxal (Miller & Gerrard, 2005).

For incubation with both glutaraldehyde and methylglyoxal, visual inspection of the samples throughout the incubation showed that there were precipitates at the bottom of the Eppendorf tubes for the more highly crosslinked samples. The precipitate

was quantified and found e.g. to reach about 1 mg/ml with 300 mM methylglyoxal, compared to those without cross-linking reagents (Supplementary Fig. 1a and b).

The results of the SDS–PAGE analysis were consistent with: (i) the proteins being unreactive to Maillard chemistry compared to those studied in previous research; (ii) amino acid side chains being derivatised by glutaraldehyde and methylglyoxal, but the adducts not proceeding to form crosslinks; or (iii) that intramolecular crosslinks were formed preferentially to intermolecular crosslinks. To distinguish between the first scenario and the other two, the two most Maillard reactive amino acid residues, lysine and arginine, were examined during the reaction.

3.3. Loss of lysine residues

The lysine residues in cocoa protein extracted with SDS-containing buffer, and buffer containing urea were analysed using the established OPA assay (Gerrard et al., 2003a) and remained unchanged when they were incubated with 0–10 mM glutaraldehyde and methylglyoxal (Fig. 3). However, lysine residues started to decline significantly after the concentration of the cross-linking reagent exceeded 20 mM. Lysine residue loss was more pronounced with glutaraldehyde than methylglyoxal (Fig. 3). In contrast, glutaraldehyde had an immediate reaction with wheat proteins *in vitro*, although the reaction was least effective in the gliadin fraction (Gerrard et al., 2003a). Marquie (2001) also reported that glutaraldehyde reacted effectively with lysine residues in cotton seed protein.

3.4. Loss of arginine residues

Available arginine residues were analysed in an analogous manner to that used for the lysine residues and were also found to be significantly reduced when the samples were incubated with glutaraldehyde and methylglyoxal (Fig. 4). Arginine residues were completely removed by methylglyoxal, consistent with the known reactivity of this compound (Lederer & Kläiber, 1999). Fig. 4 demonstrates that complete loss of measurable arginine residue occurred at concentrations of glutaraldehyde and methylglyoxal higher than 50 and 5–10 mM, respectively, in both buffers. This

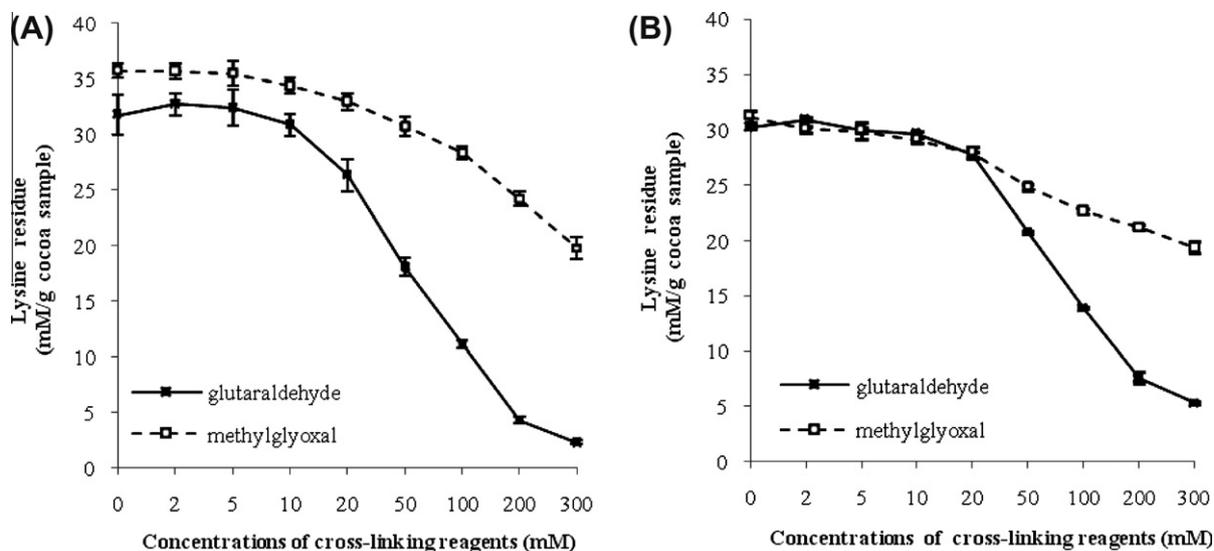


Fig. 3. Lysine residue analysis of cocoa proteins extracted with SDS-containing buffer (A) and urea (B), incubated with various concentrations of glutaraldehyde and methylglyoxal. Error bars represent the standard error of the mean of six replicate measurements.

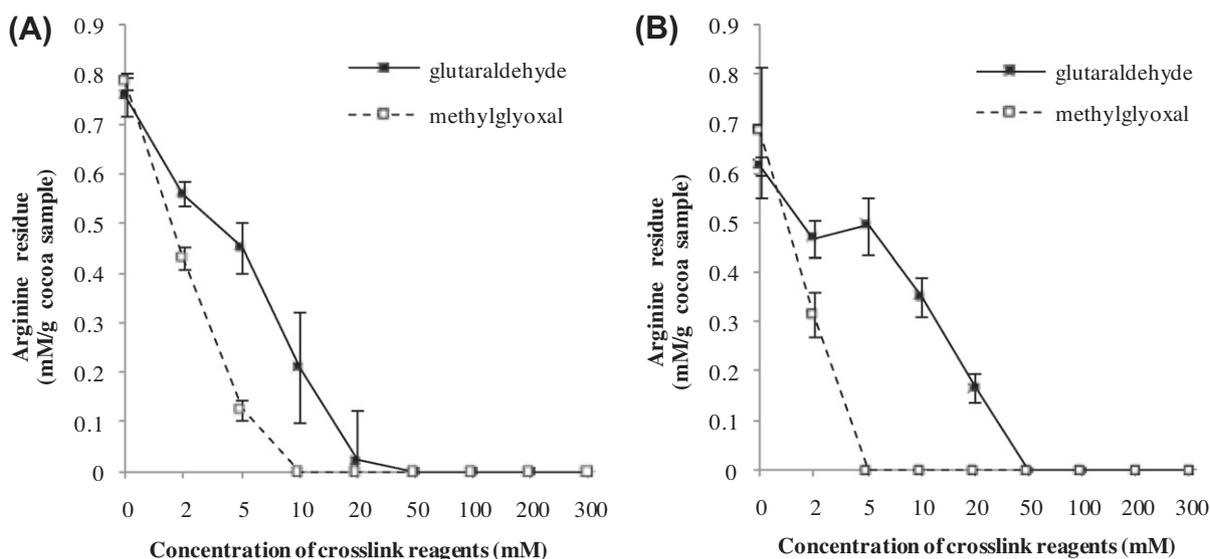


Fig. 4. Arginine analysis of cocoa proteins extracted with SDS-containing buffer (A) and urea (B), incubated with various concentrations of glutaraldehyde and methylglyoxal. Error bars represent the standard error of the mean of six replicate measurements.

points towards the lack of crosslinking observed being due to non-crosslinking modifications on the arginine residues.

3.5. Polyphenol content

It has been previously suggested that the presence of polyphenols can reduce the extent of Maillard chemistry in cocoa beans during roasting (Misnawi, Jinap, & Jamilah, 2004; Noor-Soffalina, Jinap, Nazamid, & Nazimah, 2009). Totlani and Peterson (2005) reported that epicatechin reacted directly with sugar fragments such as methylglyoxal. The total polyphenol content of the extracted cocoa samples was therefore measured during the course of the reaction (Supplementary Fig. 2) and found to be present in significant concentrations. This may also explain some of the smearing witnessed in the SDS-PAGE, if the polyphenol complexes with the proteins, as suggested by Kusuda, Hatano, and Yoshida (2006). This provides a further possible explanation for the limited extent of

crosslinking observed in this study, although more work would be required to substantiate this hypothesis.

4. Conclusion

This study revealed that much of the protein in non-roasted cocoa beans cannot be extracted using aqueous buffers. Incubation of the extracted proteins with various concentrations of glutaraldehyde and methylglyoxal led to a reduction in both lysine and arginine residues and limited crosslinking at high concentrations of crosslinking reagent. The loss of arginine residues was particularly marked. These data suggest that the cross-linking reagents derivatised amino acid side-chains, but did not proceed to full intermolecular protein cross-linking.

The total amount of soluble polyphenols in the dried fat-free mass of fresh cocoa beans is 15–20%. The major groups of polyphenols in cocoa beans are catechins or flavan-3-ols (ca. 37%),

anthocyanins (ca. 4%) and proanthocyanidins (ca. 58%) (Wollgast & Anklam, 2000). Polyphenols have been reported to reduce the Maillard reaction of cocoa beans during roasting (Misnawi et al., 2004; Noor-Soffalina et al., 2009; Totlani & Peterson, 2006). It is suggested that that polyphenols in cocoa interfere with the Maillard reaction, which limits the opportunities for exploiting Maillard crosslinking chemistry in cocoa bean processing.

Acknowledgement

The authors would like to thank Thailand's Commission of Higher Education, Ministry of Education for the financial support of this project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2012.02.189.

References

- Abecia-Soria, L., Pezoa-Garcia, N. H., & Amaya-Farfan, J. (2005). Soluble albumin and biological value of protein in cocoa (*Theobroma cacao* L.) beans as a function of roasting time. *Journal of Food Science*, 70, S294–S298.
- Adeyeye, E. I., Akinyeye, R. O., Ogunlade, I., Olaofe, O., & Boluwade, J. O. (2010). Effect of farm and industrial processing on the amino acid profile of cocoa beans. *Food Chemistry*, 118, 357–363.
- Amin, I., Jinap, S., & Jamilah, B. (1997). Vicilin-class globulins and their degradation during cocoa fermentation. *Food Chemistry*, 59, 1–5.
- AOAC 970.22 (2000). Official methods of analysis of AOAC International (17th ed.). Gaithersburg, MD, USA.
- Aremu, C. Y., Agiang, M. A., & Ayatse, J. O. I. (1995). Nutrient and antinutrient profiles of raw and fermented cocoa bean. *Plant Foods for Human Nutrition*, 48, 217–223.
- Bigi, A., Cozzani, G., Panzavolta, S., Rubini, K., & Roveri, N. (2001). Mechanical and thermal properties of gelatin films at different degrees of glutaraldehyde crosslinking. *Biomaterials*, 22, 763–768.
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Analytical Biochemistry*, 72, 248–254.
- Buyukpamukcu, E., Goodall, D. M., Hansen, C.-E., Keely, B. J., Kochhar, S., & Wille, H. (2001). Characterization of peptides formed during fermentation of cocoa bean. *Journal of Agricultural and Food Chemistry*, 49, 5822–5827.
- Chatterjee, M., & Mandal, D. K. (2003). Kinetic analysis of subunit oligomerization of the legume lectin soybean agglutinin. *Biochemistry*, 42, 12217–12222.
- de Brito, E. S., García, N. H. P., & Amâncio, A. C. (2002). Effect of polyphenol oxidase (PPO) and air treatments on total phenol and tannin content of cocoa nibs. *Ciência e Tecnologia de Alimentos*, 22, 45–48.
- de Brito, E. S., García, N. H. P., Gallão, M., Cortelazzo, A. L., Fevereiro, P. S., & Braga, M. R. (2000). Structural and chemical changes in cocoa (*Theobroma cacao* L.) during fermentation, drying and roasting. *Journal of the Science of Food and Agriculture*, 81, 281–288.
- Fayle, S. E., Healy, J. P., Brown, P. K., Reid, E. A., Gerrard, J. A., & Ames, J. M. (2001). Novel approaches to the analysis of the Maillard reaction of proteins. *Electrophoresis*, 22, 1518–1525.
- Gerrard, J. A. (2002). Protein–protein crosslinking in food: Methods, consequences, applications. *Trends in Food Science Technology*, 13, 391–399.
- Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2002). Maillard crosslinking of food proteins I: The reaction of glutaraldehyde, formaldehyde and glyceraldehyde with ribonuclease. *Food Chemistry*, 79, 343–349.
- Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2003a). Maillard crosslinking of food proteins III: The effects of glutaraldehyde, formaldehyde and glyceraldehyde upon bread and croissants. *Food Chemistry*, 80, 45–50.
- Gerrard, J. A., Brown, P. K., & Fayle, S. E. (2003b). Maillard crosslinking of food proteins II: The reaction of glutaraldehyde, formaldehyde and glyceraldehyde with wheat proteins *in vitro* and *in situ*. *Food Chemistry*, 80, 35–43.
- Granvogel, M., Bugar, S., & Schieberle, P. (2006). Formation of amines and aldehydes from parent amino acids during thermal processing of cocoa and model systems: New insights into pathways of the Strecker reaction. *Journal of Agricultural and Food Chemistry*, 54, 1730–1739.
- Hashim, P., Selamat, J., Muhammad, S. K. S., & Ali, A. (1998). Changes in free amino acid, peptide-N, sugar and pyrazine concentration during cocoa fermentation. *Journal of the Science of Food and Agriculture*, 78, 535–542.
- Kaye, Y. K., Easa, A. M., & Ismail, N. (2001). Reducing weight loss of retorted soy protein tofu by using glucose- and microwave-pre-heating treatment. *International Journal of Food Science & Technology*, 36, 387–392.
- Kratzer, U., Frank, R., Kalbacher, H., Biehl, B., Wöstemeyer, J., & Voigt, J. (2009). Subunit structure of the vicilin-like globular storage protein of cocoa seeds and the origin of cocoa- and chocolate-specific aroma precursors. *Food Chemistry*, 113, 903–913.
- Kusuda, M., Hatano, T., & Yoshida, T. (2006). Water-soluble complexes formed by natural polyphenols and bovine serum albumin: Evidence from gel electrophoresis. *Bioscience, Biotechnology, Biochemistry*, 70, 152–160.
- Lederer, M. O., & Klaiber, R. G. (1999). Cross-linking of proteins by Maillard processes: Characterization and detection of lysine–arginine cross-links derived from glyoxal and methylglyoxal. *Bioorganic & Medicinal Chemistry*, 7, 2499–2507.
- Lerceteau, E., Rogers, J., Pétiard, V., & Cruzillat, D. (1999). Evolution of cacao bean proteins during fermentation: A study by two-dimensional electrophoresis. *Journal of the Science of Food and Agriculture*, 79, 619–625.
- Marquie, C. (2001). Chemical reactions in cottonseed protein cross-linking by formaldehyde, glutaraldehyde, and glyoxal for the formation of protein films with enhanced mechanical properties. *Journal of Agricultural and Food Chemistry*, 49, 4676–4681.
- Meade, S. J., Miller, A. G., & Gerrard, J. A. (2003). The role of dicarbonyl compounds in non-enzymatic crosslinking: A structure–activity study. *Bioorganic & Medicinal Chemistry*, 11, 853–862.
- Miller, A. G., & Gerrard, J. A. (2005). Assessment of protein function following cross-linking by α -dicarbonyls. *Annals of the New York Academy of Sciences*, 1043, 195–200.
- Misnawi & Ariza, B. T. S. (2011). Use of gas chromatography–olfactometry in combination with solid phase micro extraction for cocoa liquor aroma analysis. *International Food Research Journal*, 18, 829–835.
- Misnawi Jinap, S., Jamilah, B., & Nazamid, S. (2004). Effect of polyphenol concentration on pyrazine formation during cocoa liquor roasting. *Food Chemistry*, 85, 73–80.
- Mitchell, J. R., & Hill, S. E. (1995). The use and control of chemical reactions to enhance the functionality of macromolecules in heat-processed foods. *Trends in Food Science Technology*, 6, 219–224.
- Noor-Soffalina, S. S., Jinap, S., Nazamid, S., & Nazimah, S. A. H. (2009). Effect of polyphenol and pH on cocoa Maillard-related flavor precursors in a lipidic model system. *International Journal of Food Science and Technology*, 44, 168–180.
- Redgwell, R. J., Trovato, V., & Curti, D. (2003). Cocoa bean carbohydrates: Roasting-induced changes and polymer interactions. *Food Chemistry*, 80, 511–516.
- Schmitt, A., Schmitt, J., Münch, G., & Gasic-Milencovic, J. (2005). Characterization of advanced glycation end products for biochemical studies: Side chain modifications and fluorescence characteristics. *Analytical Biochemistry*, 338, 201–215.
- Silva, C. J. S. M., Sousa, F., Gübitz, G., & Cavaco-Paulo, A. (2003). Chemical modifications on proteins using glutaraldehyde. *Food Technology and Biotechnology*, 42, 51–56.
- Sun, Y., Hayakawa, S., & Izumori, K. (2004). Antioxidative activity and gelling rheological properties of dried egg white glycated with a rare ketohexose through the Maillard reaction. *Journal of Food Science*, 69, C427–C434.
- Totlani, V. M., & Peterson, D. G. (2005). Reactivity of epicatechin in aqueous glycine and glucose Maillard reaction models: Quenching of C2, C3, and C4 sugar fragments. *Journal of Agricultural and Food Chemistry*, 53, 4130–4135.
- Totlani, V. M., & Peterson, D. G. (2006). Epicatechin carbonyl-trapping reactions in aqueous Maillard systems: Identification and structural elucidation. *Journal of Agricultural and Food Chemistry*, 54, 7311–7318.
- Uchida, K., Khor, O. T., Oya, T., Osawa, T., Yasuda, Y., & Miyata, T. (1997). Protein modification by a Maillard reaction intermediate methylglyoxal. Immunochemical detection of fluorescent 5-methylimidazolone derivatives *in vivo*. *FEBS Letters*, 410, 313–318.
- Voigt, J., Biehl, B., & Wazir, S. K. S. (1993). The major seed proteins of *Theobroma cacao* L. *Food Chemistry*, 47, 145–151.
- Wollgast, J., & Anklam, E. (2000). Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Research International*, 33, 423–447.
- Yasir, S. B., Sutton, K. H., Newberry, M. P., Andrews, N. R., & Gerrard, J. A. (2007). The impact of Maillard cross-linking on soy proteins and tofu texture. *Food Chemistry*, 104, 1502–1508.
- Zak, D. L., & Keeney, P. G. (1976). Changes in cocoa proteins during ripening of fruit, fermentation, and further processing of cocoa beans. *Journal of Agricultural and Food Chemistry*, 27, 483–486.