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Activation of remaining key enzymes in dried under-fermented cocoa beans and its effect on aroma precursor formation

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Abstract

Incubation-activation of remaining key enzymes in dried under-fermented cocoa beans and its effect on aroma precursor formation has been studied using defatted unfermented and partly fermented cocoa bean powders. Results of the study showed that aspartic endoprotease, carboxypeptidase and invertase were significantly inactivated during fermentation and drying, and the effect of fermentation was significantly lower than that of drying. The enzyme activities remaining in these beans were still sufficient to carry out enzymatic reaction during incubation. Peptide patterns, resulting from incubation of unfermented and partly fermented beans powders, were quite similar to the well-fermented patterns. Meanwhile, free amino acid concentrations of the unfermented beans were significantly increased during the first 4 h of incubation and then remained constant; however, with partly fermented beans, the formation continued and the hydrophobic and total free amino acid concentrations reached the value of well-fermented beans after 24 h of incubation. Reducing sugar concentrations of both unfermented and partly fermented cocoa beans could reach the level of well-fermented beans by incubation.

Keywords: Cocoa; Aroma; Peptide; Amino acid; Sugar; Aspartic endoprotease; Carboxypeptidase; Invertase

1. Introduction

Aroma precursors in cocoa beans, which include free amino acids, peptides and reducing sugars, develop into coco-specific aroma through Maillard reactions during roasting (Barel, Leon, & Vincent, 1985; Mohr, Landschreiber, & Severin, 1976). Nicolau, de Revel, Bertrand, and Maujean (2000) and Hofmann and Schieberle (2000) have demonstrated the formation of flavour compounds involving amino acids and reducing sugars in the Maillard reaction using a model system.

During cocoa fermentation, proteolysis, catalyzed by aspartic endoprotease and carboxypeptidase, gives rise to amino acids and oligopeptides (Biehl, Wewetzer, & Passern, 1982; Puziah, Jinap, Sharifah, & Asbi, 1998a, 1998b). The aspartic endoprotease from cocoa beans cleaves protein substrate preferentially at hydrophobic amino acid residues, to produce oligopeptides having hydrophobic amino acid residues at their carboxy terminal ends. Carboxypeptidase plays an important role in converting hydrophobic oligopeptides to cocoa-specific aroma precursors, namely hydrophilic oligopeptides and hydrophobic free amino acids, which are required for the formation of the typical cocoa aroma components in the presence of reducing sugar upon roasting (Voigt, Biehl, Heinrichs, Kamaruddin, Marsoner, & Hugi, 1994).

The predominant sugars in cocoa beans are sucrose, fructose and glucose. In unfermented cocoa beans, sucrose was found to be present in significant concentration (18.8 g kg^{-1}), comprising about 95% of the total sugars (Puziah et al., 1998a). During cocoa fermentation, sucrose was almost completely hydrolysed to fructose and glucose by invertase present in the beans (Puziah et al., 1998a, 1998b; Rohan & Stewart, 1967).

Studies of enzyme activities during processing by Hansen, del Olmo, and Burri (1998) showed that aminopeptidase, invertase (in pulp), and polyphenol oxidase were strongly inactivated during fermentation; carboxypeptidase was partly inactivated, whereas endoprotease and glycosidases remained active. Polyphenol oxidase was the only enzyme strongly inactivated during the drying process. Subsequent study by Hansen, Manez, Burri, and Bousbaine (2000) found that cocoa

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flavour potential was not necessarily correlated with high activities of those endogenous enzymes. This paper discusses the effects of incubation or activation of remaining key enzymes, namely aspartic endoprotease, carboxypeptidase and invertase, in dried-unfermented and partly fermented cocoa beans, on aroma precursor development.

2. Materials and methods

2.1. Cocoa beans preparation

Cocoa beans used in the study were F1 hybrid (*GC7* Vs. *SCA6*/*SCA12*) obtained from Kotta Blater Estate, Indonesia. Fermentation was carried out using a wooden box of 2 tonnes capacity of 100 cm width, 200 cm length and 90 cm depth. The fermentation mass was turned manually daily by transferring from one box to another. Cocoa beans of 0 day (unfermented), 1 day, 2 days' and 5 days' fermentation were collected and sun-dried until they reached 7% moisture content. Dried beans were then peeled, lyophilized, ground to size <0.5 mm, and defatted using petroleum benzene (b.p. 40–60 °C).

2.2. Design and statistical analysis

The studies were carried out in single factor, using complete randomized design (CRD) with four replications. The treatments were time of incubation, consisting of 0, 4 and 16 h for dried-unfermented cocoa beans, and 0, 16 and 24 h for dried partly (2 days) fermented beans. Dried well fermented (5 days) beans were used as control. Data obtained were analyzed using SAS package software (SAS Institute, Inc., 1996, Version 6.12). Statistical significance was assessed by analyses of variance and significant differences were detected using Duncan's multiple range test.

2.3. Incubation

Defatted cocoa powder (5 g) was suspended in 250 ml of 10 mM acetate buffer, pH 5.5. Suspensions were then incubated at 45 °C in an orbital shaker incubator YIH DER LM 510R at 150 rpm. After incubation, the suspension was quickly lyophilized at <-20 °C for 16 h, freeze-dried at pressure $<133\times10^{-3}$ mbar using a Labconco Freezone 6 apparatus, and then ground in a Braun ZK 100 cutter blender to 40 mesh.

2.4. Enzyme activities assay

2.4.1. Acetone dry powder (AcDP)

Acetone dry powder was prepared using the method of Hansen et al. (1998). The dried powder was extracted 5 times with 80% chilled acetone and 3 times with 100% chilled acetone. The extraction used 3 g powder and 30 ml solvent in a 50 ml polypropylene centrifuge tube. The mixture was homogenized using a Polytron Homogenizer (15 s at low speed, 4 °C), followed by centrifugation in a Kubota 7800 instrument at 4 °C for 5 min at 10,000 ***g***. The remaining solvents were discarded by freeze-drying and the final pellet was ground to a fine dry powder with mortar and pestle. The dry powder was then stored at <-20 °C, and used as enzyme source for determination of enzyme activities.

2.4.2. Preparation of enzyme solution

Enzyme solution was prepared by using the method of Amin, Jinap and Jamilah (1998) with slight modifications. The AcDP was suspended in cold 0.2 M sodium phosphate buffer pH 7.5 in the ratio 1:50 w/v and mixed for 10 min at 5 °C. After mixing, the suspension was centrifuged in a Kubota 7800 instrument at 10,000 ***g*** for 30 min at 4 °C. The supernatant was dialyzed against the same buffer at 5 °C for 2 days with daily change of buffer. The dialyzed solution was centrifuged at 10,000 ***g*** for 30 min and the supernatant was used for the determination of enzyme activities.

2.4.3. Aspartic endoprotease assay

Five hundred microlitres of the enzyme solution were incubated at 45 °C for 30 min in a reaction mixture containing 20 mg of bovine serum albumen and 2.0 ml of 0.2 M phosphate buffer, adjusted to pH 3.5 by addition of acetic acid. The reaction was terminated by adding 0.5 ml of 20% chilled trichloroacetic acid solution and then centrifuged at 10,000 ***g*** for 15 min (Amin et al., 1998; Hansen et al., 1998). The amount of proteolytic products was determined by ninhydrin reaction, as described by Hansen et al. (1998). One unit of aspartic endoprotease is the amount of enzyme needed to liberate 1 μmol amino groups per min under the above conditions. L-Leucine (Sigma-Aldrich Co.) was used as the standard (Amin et al., 1998; Voigt, Biehl et al., 1994).

2.4.4. Carboxypeptidase activity

A 1 ml enzyme solution was pre-incubated in an ice-bath in the presence of 10 μg/ml pepstatin A for 1 h to inhibit the aspartic endoprotease (Amin et al., 1998; Voigt, Biehl et al., 1994). The aliquot (0.5 ml) was added to 0.5 ml of 0.2 M sodium phosphate buffer, pH 5.8, containing 5 mM Z–Phe–Leu as a substrate. The substrate was prepared from 125 mM stock solution in methanol. The mixture was incubated at 45 °C for 30 min. The reaction was terminated by adding 0.5 ml of 20% cold trichloroacetic acid solution and centrifuged at 3000 ***g*** for 20 min. The amount of amino groups released was determined by ninhydrin reaction, as described by Hansen et al. (1998). One unit of carboxypeptidase is the amount of enzyme needed to release 1 μmol leucine per min at pH 5.8 and 45 °C.

2.4.5. Invertase activity

The enzyme solution (0.5 ml) was added to 1.84 ml of 0.2 M sodium phosphate buffer pH 4.5 and 0.16 ml of 300 mM sucrose in phosphate buffer pH 4.5. The mixture was incubated for 3 h at 50 °C with gentle shaking, and then heated for 5 min at 100 °C to stop the reaction. The amount of sucrose inverted was measured by HPLC, as described in the sugars determination procedure. Rhamnose was used as an internal standard for the determination. One unit of invertase is the amount of enzyme needed to invert 1 μmol sucrose per min under the above conditions.

2.5. Peptides pattern analysis

One gramme of defatted cocoa powder was diluted in 10 ml of 70% (v/v) methanol and homogenized for 5 min. The suspension was then centrifuged at 22,000 ***g*** at 4 °C for 20 min. The supernatant was collected and the methanol was removed under vacuum at 40 °C by means of a rotary evaporator. Finally, the aqueous solution was passed through activated charcoal and filtered through a 0.45-μm membrane filter prior to HPLC application.

The HPLC analysis of peptides was performed by the method of Voigt, Biehl, et al. (1994), using a Waters Associates Model 600 Controller and model 486 Tunable Absorbance Detector equipped with an Ultraspere ODS 5μ column (6.6 mm ×25 cm). Elution of the peptides was performed at 30 °C and flow rate of 1 ml per min with 0.1% (v/v) aqueous trifluoroacetic acid for 7 min and subsequently with a linier gradient from 0 to 50% (v/v) acetonitrile containing 0.1% trifluoroacetic acid for 53 min. The eluting peptides were monitored by measuring the absorbance of the effluent at 210 nm.

2.6. SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)

SDS–PAGE analysis was carried out using the methods of Pettipher (1990) in a vertical mini slab gel (5 cm ×1 mm), using a Bio-Rad Mini Protean II cell. Two hundred milligrammes of defatted cocoa powder were added with 1.5 ml of sample buffer LAEMMLI (Sigma Chemical Co.), and then boiled in a water bath for 15 min. After cooling in an ice bath for 10 min, insoluble particles were separated by centrifugation using an Eppendorf Centrifuge 5415C at 6000 rpm for 15 min at room temperature. Ten microlitres of the supernatant were then carefully loaded into each well for gel electrophoresis. The electrophoresis was carried out at a constant current of 40 mA for stacking gel and 30 mA for separating gel. The electrophoresis was terminated when the tracking blue dye (bromophenol blue) reached 1.0 cm from the bottom of the gel.

After electrophoresis, the gel was carefully removed from the sandwiched glass plate and immersed in a staining solution. Gel was stained and destained using coomassie brilliant blue and acetic acid/methanol solution, respectively, until the background became clear. The relative molecule weights of the protein samples were estimated by comparing relative mobility (R_f) values of standard markers against R_f values of protein samples. R_f is defined as the ratio of the migration distance (mm) of the marker proteins and the migration of the bromophenol blue dye. SDS molecular weight marker (MW-SDS 70) from Sigma Chemical Co. was used as a standard.

2.7. Free amino acids determination

Free amino acids were prepared using the extraction procedure of Kirchhoff, Beihl, Ziegeler-Berghausen, Hammoor, and Lieberei (1989) and determined by the HPLC method of Cohen, Meys, and Tarvin (1990) that was modified by Puziah et al. (1998a). Seven hundred milligrammes of defatted cocoa powder plus 1.4 g polyvynylpymollydone were homogenized in 15 ml distilled water using a Polytron Homogenizer for 5 min at 0 °C, and then adjusted to pH 2.5 using glacial acetic acid. The mixture was centrifuged at 13,000 ***g*** for 15 min, and then filtered through Whatman filter paper No. 4. Filtrate obtained was then made up to 50 ml using distilled water. Internal standard of alpha-amino butyric acid (AABA) and 12 ml acetone were added to 3 ml filtrate, kept at room temperature for 30 min, and then centrifuged at 13,000 ***g*** for 15 min. Acetone was removed by nitrogen streaming.

The amino acids were converted into phenylthiocarbonyl amino acids using phenylisothiocyanate, as described by Cohen et al. (1990). The free amino acids were separated by reverse-phase HPLC, employing a Waters Pico-Tag free amino acid column 3.9×300 mm, using gradient elution. Twenty microlitres of sample extract were used. Solvent A of the gradient elution consisted of sodium acetate buffer pH 5.7 and solvent B consisted of acetonitrile:water 60:40. The gradient elution was as follows: 0 min: 100% A, 0% B; 5 min: 75% A, 25% B; 13 min: 52% A, 48% B; 13.5 min: 0% A, 100% B; 16.5 min: 0%A, 100%B; 17 min: 100% A, 0% B; 22 min: 100% A, 0% B. The free amino acids were detected using a Waters Associates model 486 Tunable Absorbance Detector at 254 nm.

Free amino acids obtained were then classified into acidic amino acids, consisting of aspartic and glutamic acids, hydrophobic amino acids, consisting of leucine, alanine, phenyl alanine, tyrosine, valine and iso leucine, and other class amino acids, consisting of proline, methionine, cysteine, lysine, serine, glycine, histidine and arginine.

2.8. Fermentation index determination

Five hundred milligrammes of defatted cocoa powder were weighed into a 125 ml conical flask before a mixture

of 50 ml of methanol:hydrochloric acid (97:3) solution was added, the mixture was cooled at 8±2 °C in a refrigerator for 16–18 h. A clear extract was obtained by filtration through a Whatman No. 1 filter paper. Fermentation index was calculated, based on the ratio of the absorbance at 460 nm to the absorbance at 530 nm (Goerieva & Tserevinov, 1979).

2.9. Sugars determination

One gramme of defatted cocoa powder was homogenized with 20 ml of 85% methanol using a Polytron Homogenizer for 5 min. Internal standard (rhamnose) was applied in this step. The mixture was then heated on a steam bath for 30 min and filtered through a Whatman No. 1 filter paper. The filtrate was subjected to a vacuum evaporator for evaporation to about 10 ml at 50 °C. The extract was then filtered through a Sep Pak C_{18} cartridge and a 0.45 μm membrane filter using a syringe.

A Waters Associates Model 600 Controller and model 410 RI Detector were used for sugar determination. Separation of sugars was accomplished using a μBondapak-NH_2 (300×3.9 mm i.d.) stainless steel tube column. The eluent was isocratic, consisting of acetonitrile:deionized water at 80:20 (v/v) and flow rate 2.5 ml per min. Ten microlitres of sample extract were used. Sucrose, glucose and fructose (Sigma-Aldrich Co.) were used as standards.

3. Results and discussion

3.1. Enzyme activities

Fermentation is a very important aspect of cocoa bean processing. Study has shown that unfermented beans do not develop any chocolate flavour when roasted and are excessively astringent and bitter (Biehl & Voigt, 1996; Puziah et al., 1998a, 1998b). Therefore, the beans are not preferred by cocoa manufacturers. To increase acceptance and flavour quality, the under-fermented beans are usually blended with well-fermented beans. Under-fermented refers to the beans that are dried (under sun-drying) without fermentation or fermented for 1–2 days; the beans were usually produced by smallholders.

Evaluation of the remaining key enzymes in the dried under-fermented cocoa beans (Table 1) shows that the drying process significantly inactivated protease and invertase in cocoa beans. The inactivation effect on fermentation (2 days) was lower than the drying effect. The remaining aspartic endoprotease activities in dried-unfermented and partly fermented cocoa beans were 34 and 31% of the activity in fresh beans, respectively, while those of carboxypeptidase activity were 20 and 16%. Inactivation effect of drying and fermentation was higher on the invertase than either the aspartic endoprotease or carboxypeptidase. It was found that the remaining invertase activities in dried-unfermented and partly fermented cocoa beans were 19 and 7% of the activity in fresh beans, respectively.

The above remaining key enzyme activities were high enough to form aroma precursors by incubation-activation. According to previous studies by Hansen et al. (1998, 2000), enzymatic reaction in cotyledon could continue during fermentation although to a limited extent.

Table 1 also shows that the remaining enzymes partially lost their activity immediately after the reaction started. This may be due to the fact that polyphenols bind to the protein-site of the enzymes during incubation. Dried-unfermented and partly fermented cocoa beans are rich in polyphenols, comprising 12–18% of their dry weight (Kim & Keeney, 1984). Lopez (1986) stated that the products of polyphenol oxidation inactivate hydrolytic enzymes during cocoa bean processing; therefore, if the oxidation occurs immediately after the death of the beans, the cocoa produced would be deficient in chocolate flavour. According to Hagerman (1992), the phenolic hydroxyl group is an excellent hydrogen bond donor, and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone. Bartolome, Estrelia, and Hernandez (2000), Peleg, Gacon, Schlich, and Noble (1999) and Kallithraka, Bakker, and Clifford (2001) have successfully proved the interaction between polyphenols and protein in a model system. Katternberg and Kemmink (1993) also found that enzymatic oxidation of polyphenols produces quinones; these are very reactive agents, and they can react further with amino acids and proteins, or polymerize with each other to form high molecular weight products, the so-called "condensed tannins", whereas at molecular weight above 3000, they form complexes with protein through hydrogen bonding. Peleg et al. (1999) reported that polymerization of polyphenols increases the intensity of formation of phenol–protein complex.

3.2. Peptide patterns and SDS–PAGE

Peptide patterns of the extract from the defatted cocoa bean powder, after incubation treatments, are presented in Figs. 1 and 2. Using reverse-phase HPLC, most of the more hydrophilic peptides were first eluted followed by the less hydrophilic ones (Voigt, Biehl et al., 1994; Voigt, Wrann, Heinrichs, & Biehl, 1994).

Fig. 1A–C display significant changes of peptide patterns during incubation of the defatted dried unfermented cocoa bean powders. Prior to incubation, dried-unfermented cocoa beans exhibited dominant peptides at retention times of 18–25 min, and smaller quantities at retention times of 4–8 min. Incubation treatment

Table 1
Effect of incubation of defatted dried unfermented and partly fermented cocoa beans powder on proteases and invertase activities

Incubation	Activity (units g^{-1} DW)	Activity lost[a] during incubation (% h^{-1})	Remaining activity (percent to fresh beans activity)
Aspartic endoprotease			
Fresh beans	7.37±0.135	n.a	100.0
Unfermented beans			
0 h	2.47±0.167	0.0	33.5
4 h	0.721±0.050	17.7	9.8
16 h	0.507±0.076	5.0	6.7
Partly fermented beans			
0 h	2.26±0.147	0.0	30.6
16 h	0.532±0.093	4.8	7.2
24 h	0.503±0.068	3.2	6.8
Well-fermented beans	trace	n.a	n.a
Carboxypeptidase			
Fresh beans	2.07±0.243	n.a	100.0
Unfermented beans			
0 h	0.413±0.045	0.0	19.9
4 h	0.184±0.043	13.8	8.9
16 h	trace	n.a	n.a
Partly fermented beans			
0 h	0.336±0.041	0.0	16.2
16 h	0.095±0.031	4.5	4.6
24 h	trace	n.a	n.a
Well fermented beans	trace	n.a	n.a
Invertase			
Fresh beans	0.106±0.010	n.a	100
Unfermented beans			
0 h	0.020±0.007	0.0	19.2
4 h	trace	n.a	n.a
16 h	trace	n.a	n.a
Partly fermented beans			
0 h	0.008±0.001	0.0	7.2
16 h	trace	n.a	n.a
24 h	trace	n.a	n.a
Well-fermented beans	trace	n.a	n.a

[a] losing activity $= \frac{(\text{activity before incubation(unit)} - \text{activity after incubation (unit)})}{\text{activity before incubation(unit)} \times \text{time of incubation (h)}} \times 100\%$

significantly reduced the peptides, at retention times of 18–25 min, to smaller quantities (Fig. 1B and C). Peptides at retention times of 4–8 min are modified first at 4 h and then reformed at 16 h of incubation. Reduction of peptides at retention times of 18–25 min is suggested to correlate with activation of carboxypeptidase. Setting temperature of incubation to 45 °C and pH 5.5 increased enzyme solubility and optimized its activity. The activity of carboxypeptidase is optimum at pH 5.8 and temperature at 45–50 °C (Biehl & Voigt, 1994). Carboxypeptidase plays an important role in converting hydrophobic oligopeptides to hydrophilic oligopeptides and hydrophobic free amino acids (Voigt, Biehl et al., 1994).

Peptide patterns of the dried-unfermented cocoa beans powder after 4 h of incubation (Fig. 1B) are relatively similar to those of well-fermented beans (Fig. 1D). Meanwhile, after 16 h of incubation (Fig. 1C), the pattern at retention time of 18–25 min was very low. This finding showed that the proteolysis progressively continued during the incubation.

In the dried partly-fermented cocoa beans, the results showed that peptides at retention times of 18–25 min slowly decreased during incubation; there were clear indications of peptides at retention times of 4–8 min. The slower peptide degradations in the dried partly fermented beans compared to dried-unfermented beans may correlate with their greater oligopeptide degradation (free amino acid formation; see discussion on free amino acids). Formation of the hydrophobic oligopeptides is the result of aspartic endoprotease activity, in which the peptides were then immediately converted

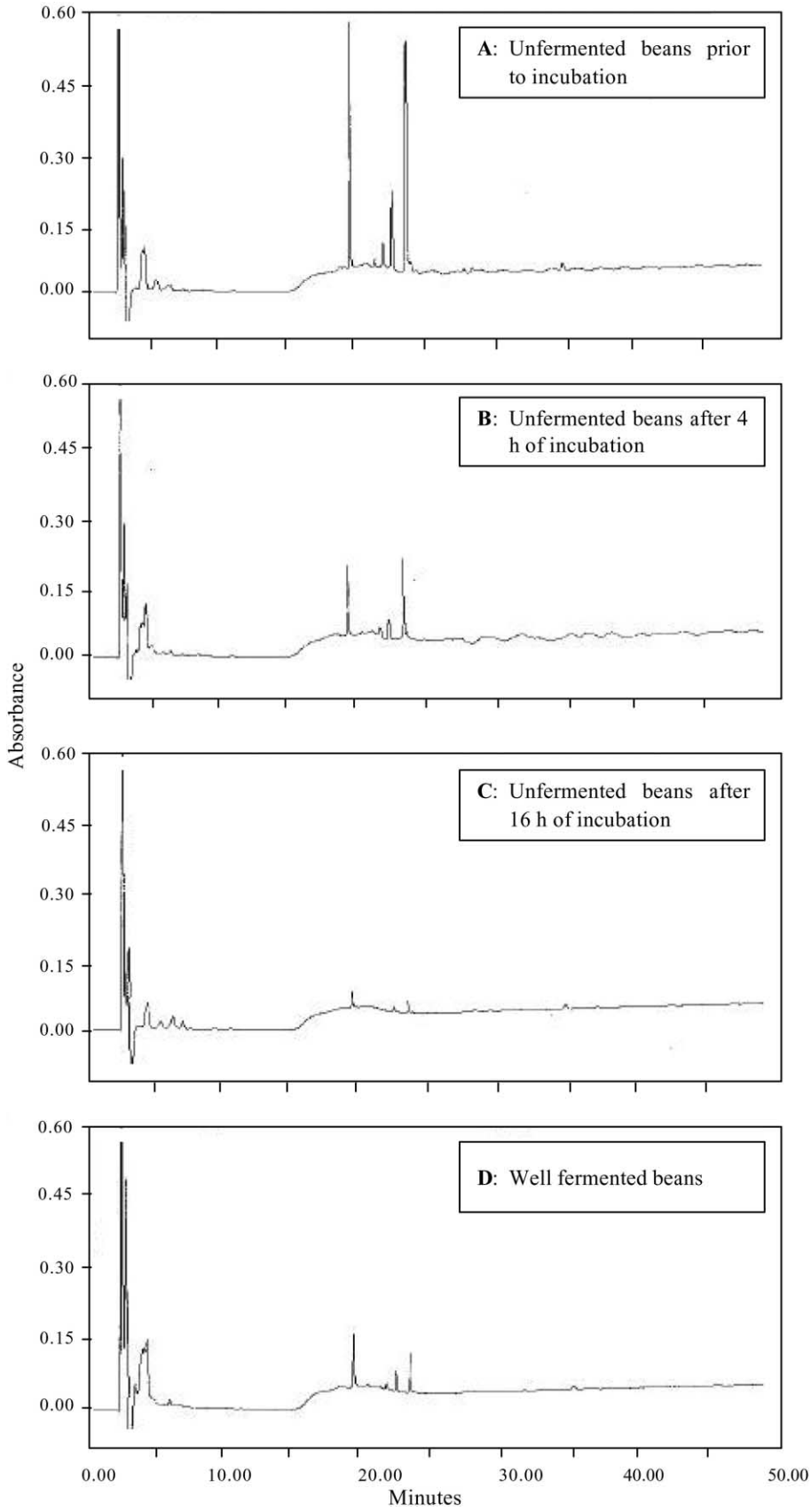

Fig. 1. Reversed-phase HPLC analysis of peptide mixtures obtained from incubation of defatted-unfermented and well-fermented cocoa bean powders.

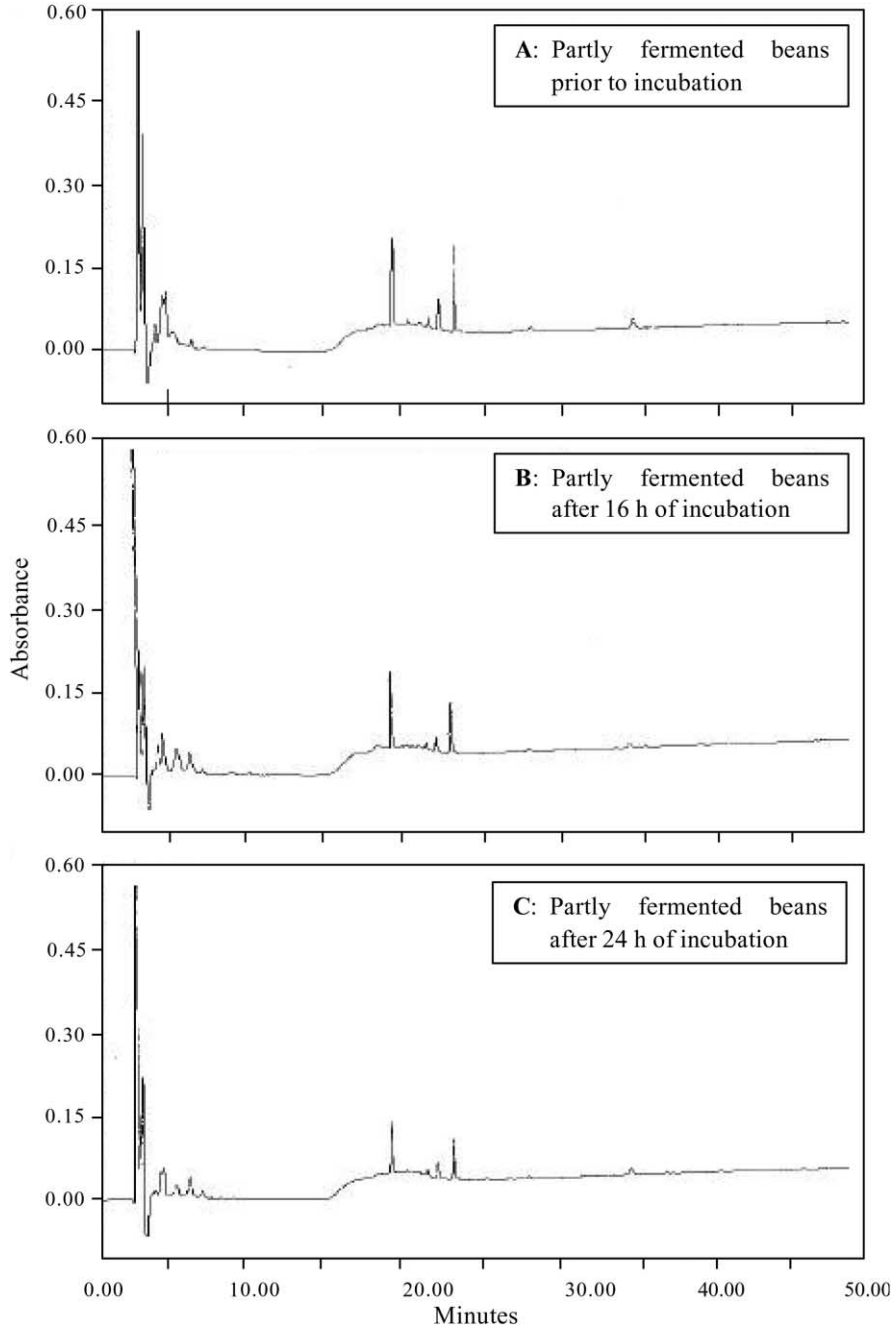

Fig. 2. Reversed-phase HPLC analysis of peptide mixtures obtained from incubation of defatted partly fermented cocoa bean powders.

to hydrophobic free amino acids and hydrophilic oligopeptides, by the action of carboxypeptidase. According to Biehl, Heinrichs, Ziegeler-Berghausen, Hammoor, and Senyuk (1993), Voigt, Biehl et al. (1994) and Voigt, Voigt, Heinrichs, Wrann, and Biehl (1994), aspartic endoprotease splits the storage protein chain, preferentially the protein chain at the site of hydrophobic amino acids, resulting in the generation of hydrophobic oligopeptides with carboxy terminal ends. Subsequently, carboxypeptidase converts the hydrophobic oligopeptides to cocoa-specific aroma precursors, namely hydrophilic oligopeptides and hydrophobic free amino acids.

SDS–PAGE results in Fig. 3 confirm the above results, and the globulin fractions, in both defatted-unfermented and partly fermented cocoa beans powders, were significantly reduced during the incubation. The major protein fractions in cocoa beans are an albumin with a molecular size of 19–21 kDa and a globulin with molecular sizes of 47–48, 31–39 and 14.5 kDa (Amin, Jinap, & Jamilah, 1997; Voigt, Biehl, & Kamaruddin, 1993). The globulin (vicilin-class globulin) fraction

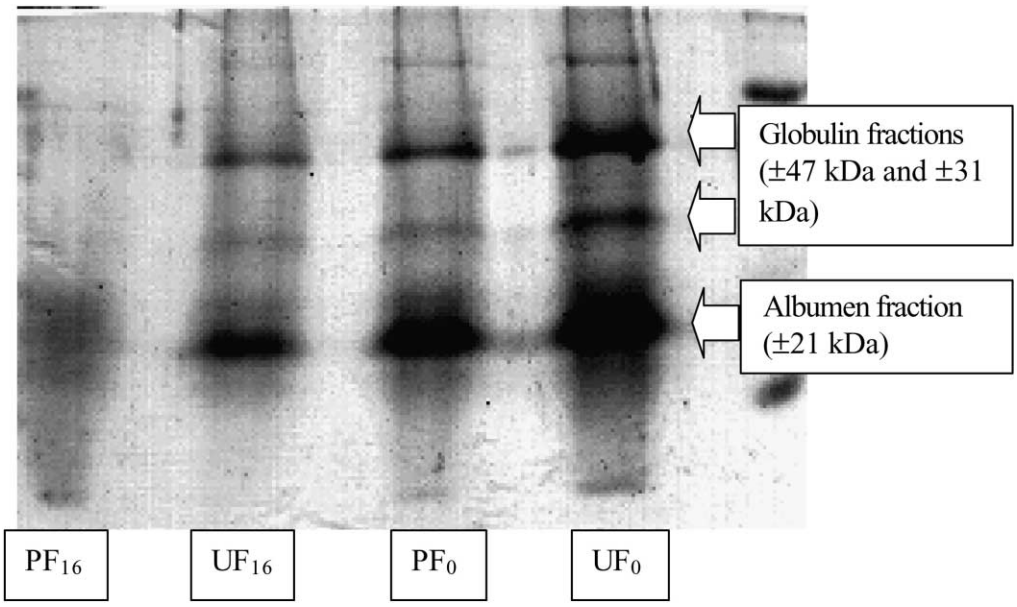

Fig. 3. SDS–PAGE analysis of defatted unfermented cocoa bean powder prior to and after 16 h of incubation (UF_0 and UF_{16}, respectively) and partly fermented cocoa beans powder prior to and after 16 h of incubation (PF_0 and PF_{16}, respectively).

is quantitatively degraded during fermentation by cocoa endoproteinase, followed by carboxypeptidase, resulting in the production of aroma precursors such as oligopeptides and free amino acids (Biehl et al., 1982).

3.3. Free amino acids

Table 2 shows the effects of incubation on free amino acids in defatted powder of dried-unfermented and partly-fermented cocoa beans. Incubation of dried-unfermented cocoa bean powder shows a significant increase in the formation of acidic, hydrophobic, other (non-acidic, non-hydrophobic) class and total free amino acids. The incubation treatments possibly activated the remaining proteases in unfermented beans to degrade protein of the beans, resulting in the accumulation of free amino acids; however, at the end of incubation, the concentrations of free amino acids did not reach the value of well fermented cocoa beans, except for acidic amino acids. Free amino acid concentrations of unfermented cocoa beans, after 16 h of incubation, were 1.17, 5.09, 0.332 and 11.3 g kg^{-1}, respectively, for acidic, hydrophobic, other classes and total free amino acids, while these of well-fermented cocoa beans were 0.461, 6.98, 3.86 and 11.3 g kg^{-1}.

Improvement of the free amino acid formation in unfermented cocoa beans powder by longer incubation may not increase the free amino acid content.The

Table 2
Effects of incubation of defatted-dried unfermented and partly-fermented cocoa bean powders on free amino acid concentrations and fermentation index[a]

Incubation	Free amino acids (g kg^{-1})				Fermentation index[b]
	Acidic	Hydrophobic	Other	Total	
Unfermented beans					
0 h	0.395b	4.16b	1.25c	5.81c	0.569d
4 h	0.320b	5.10b	2.27b	7.69b	1.76b
16 h	1.166a	5.09b	0.332d	7.59bc	2.98a
Well fermented beans	0.461b	6.98a	3.86a	11.3a	1.58c
Partly fermented beans					
0 h	0.231c	4.05b	0.663c	4.94c	0.896c
16 h	0.237c	7.78a	1.24bc	9.25b	1.15b
24 h	0.360b	7.83a	1.51b	9.70b	1.19b
Well fermented beans	0.461a	6.98a	3.86a	11.3a	1.58a

[a] Means with same letter in the same column for unfermented or partly fermented beans are not significantly different according to Duncan's Multiple Range Test at $\alpha = 0.05$.

[b] Cocoa beans are classified as sufficiently fermented if fermentation index value > 1.0.

hydrophobic and other free amino acids, as the major free amino acids, tended to decrease after 4 h of incubation. This condition could be a limiting factor in the effort to improve flavour of unfermented cocoa beans, since free amino acids, namely hydrophobic amino acids, are aroma precursors of cocoa beans, which will develop into cocoa-specific aroma upon roasting (Barel et al., 1985; Mohr et al., 1976; Voigt, Biehl et al.,1994).

Incubation of defatted, dried partly fermented cocoa bean powder showed a similar phenomenon. Hydrophobic, other classes and total free amino acids significantly increased during incubation, whereas acidic amino acids were relatively constant. As in dried unfermented beans, the free amino acid formation was due to protease activation. Protease activity, which remained in dried partly fermented cocoa beans, was still relatively high, i.e. 31 and 16% of the original activities, respectively for aspartic endoprotease and carboxypeptise (Table 1).

The possibility of improving free amino acid content in partly fermented cocoa beans, by longer incubation, may be limited by the fact that increase of free amino acids, after 16 h of incubation, was quite slow. Calculation of the free amino acids improvement after 16 h of incubation (data from Table 2), showed only 0.015, 0.006, 0.034 and 0.056 (g kg^{-1}) h^{-1}, respectively, for acidic, hydrophobic, other and total free amino acids. As in unfermented cocoa beans, this condition could be a limiting factor in the effort to improve flavour of partly fermented cocoa beans.

Free amino acid analysis also showed that the formation of free amino acids at 16 h of incubation was much higher in the dried partly fermented beans than the dried-unfermented beans. The concentration of hydrophobic free amino acids of dried partly fermented cocoa beans also reached that of well-fermented beans upon incubation. Lower free amino acid formation in the unfermented cocoa beans than in partly fermented beans could be due to amino acid metabolism and degradation (Puziah et al., 1998a) or polyphenol-protein/amino acid interactions. For this second reason, dried unfermented cocoa beans have a relatively high quantity of polyphenols, comprised 12–18% of the dry weight (Kim & Keeney, 1984), and the remaining polyphenol oxidase would oxidize the polyphenols immediately, giving quinones, which are very reactive. These compounds can react further with amino acids and proteins, or polymerize with each other and then complex with proteins through hydrogen bonding (Katternberg & Kemmink, 1993). This finding agrees with fermentation index (FI) results, in which the value of the dried unfermented cocoa beans after 4 h of incubation was significantly higher than that of partly fermented cocoa beans, even after 24 h of incubation. Fermentation index refers to the formation of brown-yellow colour as a result of the polyphenol oxidation and cocoa beans with fermentation index value >1.0 are classified as sufficiently fermented (Shamsuddin & Dimmick, 1986). The intensive polyphenol oxidation during incubation of dried unfermented beans increases the formation of the polyphenol-protein/peptide/amino acid complex (Baxter, Lilley, Haslam, & Williamson, 1997; Bianco, Chianchio, Rescuvina, Romeo, & Uccella, 1997; Kroll & Rawel, 2001); thus protein/peptide, as proteolysis substrates, were less available, and free amino acids formed at almost the same time were bound into the polyphenol-amino acid complex.

3.4. Sugars

Incubation of both defatted dried unfermented and partly fermented cocoa beans powder caused an increase in the concentration of reducing sugars (fructose and glucose) to the level of well-fermented beans at the end of incubation (Table 3). Reducing sugar contents of well-fermented cocoa beans, unfermented cocoa

Table 3
Effects of incubation of defatted-dried unfermented and partly fermented cocoa bean powders on sugars[a]

Incubation	Sugar (g kg^{-1})			
	Fructose	Glucose	Sucrose	Fructose + glucose
Unfermented beans				
0 h	3.42c	1.13c	26.2a	4.55c
4 h	5.86b	4.97b	17.7b	10.8b
16 h	9.03a	8.41a	11.6c	17.4a
Well fermented beans	8.94a	8.27a	5.55d	17.2a
Partly fermented beans				
0 h	2.65c	0.947c	25.7a	3.59c
16 h	5.69b	5.03b	16.2b	10.7b
24 h	8.35a	6.98a	11.9c	15.3a
Well fermented beans	8.94a	8.27a	5.55d	17.2a

[a] Means with same letter in the same column for unfermented or partly fermented beans are not significantly different according to Duncan's Multiple Range Test at $\alpha = 0.05$.

beans after 16 h of incubation and partly fermented beans after 24 h of incubation were 17.2, 17.4 and 15.3 g kg^{-1}, respectively. These three values are not significantly different from each other. This indicates that the incubation treatments had activated the remaining invertase in these beans to invert sucrose into fructose and glucose. Low remaining activity of invertase, in both dried-unfermented and partly fermented beans, as shown in Table 1, and its inactivation during incubation, did not prohibit the inverting reaction. The activities of invertase remaining in dried-unfermented and partly fermented cocoa beans, were only 0.020 and 0.008 units g^{-1}, respectively, and trace amounts after the first 4 h of incubation. These findings also agree with a previous study by Hansen et al. (1998), which showed that invertase was strongly inactivated during fermentation and drying; its remaining activities in unfermented and 1 day-fermented beans, were only 0.037 and 0.011 units, respectively; and no significant activity in 2 days'-fermented beans. Meanwhile, this study also found that reducing sugar contents continuously increased throughout 5 days of fermentation. This means that the inversion of sugar could occur, despite very low invertase activity.

In contrast to the phenomenon of free amino acids formation, the formation of reducing sugars was significantly faster in the incubation of dried-unfermented beans than in dried partly fermented beans. This result was suspected to be due to the differences in invertase activity, and sugars, as the substrates of invertase activity, were not influenced by polyphenol binding-interactions as are protein and amino acids. Up to now, no proper basic research has shown any interaction of polyphenols with sugars or effect on invertase activity.

4. Conclusion

Aspartic endoprotease, carboxypeptidase and invertase were significantly inactivated during fermentation and drying of cocoa beans. The inactivation effect of fermentation was significantly lower than the drying effect. The enzyme activities remaining in dried-unfermented and partly fermented cocoa beans were still sufficient to carry out enzymatic reactions during incubation. Peptide patterns of defatted-unfermented and partly fermented bean powders after incubation were quite similar to the well fermented patterns. Free amino acid concentrations in the unfermented beans increased significantly during the first 4 h of incubation and then remained constant; while in the partly fermented beans, the formation continued and the concentration of hydrophobic and total free amino acids reached the value of well-fermented beans after 24 h of incubation. Reducing sugar concentrations of well-fermented beans could be reached by the incubation of both unfermented and partly fermented cocoa beans. These findings clearly indicate that flavour quality of dried under-fermented cocoa beans can be improved through activation of the remaining key enzymes.

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