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Vicilin-class globulins and their degradation during cocoa fermentation

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Cocoa beans were fermented for 144 h using shallow wooden boxes at ambient temperature. Two major polypeptides were found to consist of the storage protein and an albumin fraction. The storage protein comprises two vicilin fractions with molecular weights of 47.1 and 39.2 kDa, and the albumin fraction has a molecular weight of 21.1 kDa. The degradation of vicilin fractions during the course of fermentation was visually detected by sodium docecyl sulphate–polyacrylamide gel electrophoresis. The albumin fraction was found to be the most resistant to proteolysis during fermentation. At the end of fermentation, the 39.2 kDa polypeptide was completely degraded but the 47.1 kDa polypeptide was still present at low intensity. The protein concentrations of 47.1 and 39.2 kDa polypeptides decreased from 1.74 to 0.03 μg and from 0.93 to 0.02 μg, respectively. The protein concentration of 46 and 46.5 kDa polypeptides increased from 0.06 to 0.34 μg and from 0.03 to 0.23 μg, respectively. This could be due to the result of the degradation products of the 47.1 kDa polypeptide.

INTRODUCTION

Storage proteins of seed plants are utilized as sources of nitrogen, carbon and sulphur, and are accumulated during germinating and ripening stages. Cocoa beans have been reported to contain four fractions of protein, i.e. albumin, globulin, prolamin and glutelin (Zak & Keeney, 1976*a*,*b*). The globulin fractions with molecular weights of 47 and 31 kDa were shown to be the polypeptides responsible for the cocoa-flavour precursors produced by the action of endoproteinases on cocoa globulin (Biehl *et al.*, 1985). Cocoa globulin is the first seed protein known to contain only vicilin-class globulin (Spencer & Hodge, 1992). Voigt *et al.* (1993) found that the presence or absence of 2-mercaptoethanol, as a reducing agent to break disulphide bonds, did not influence the electrophoretic patterns of the cocoa globulin on SDS–PAGE. Cocoa aromas were developed from roasting the precursors of vicilin-class globulin; however, no aroma was developed from roasting the precursors of albumin and other seed globulins present in hazelnuts, coconuts and sunflowers (Voigt *et al.*, 1994*b*). The vicilin-class globulins are quantitatively degraded during fermentation into flavour precursors such as peptides and amino acids, which are important precursors for the formation of cocoa flavour through Maillard reactions during roasting (Biehl *et al.*, 1982). According to Biehl *et al.* (1985), if the pH of cotyledons is within 5.0–5.5 during fermentation, this would give higher flavour potentials than fermentation at pH 4.0–4.5. Information on the degradation of a vicilin-class globulin during cocoa fermentation is still lacking. This paper describes a study of the chemical characteristics of vicilin-class globulins of cocoa beans and their degradation during fermentation.

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MATERIALS AND METHODS

Cocoa fermentation

Ripe cocoa pods of the PBC 140 variety were obtained from Sabak Bernam, Selangor, Malaysia. The fruits were depodded and 70 kg of fresh beans were fermented at ambient temperature using shallow wooden boxes (0.78×0.78×0.78 m) for a duration of 144 h. The beans were aerated by turning them manually every 48 h. Representative samples of 1 kg each were taken from the fermenting box at 0 h, and every 24 h during fermentation. The pH was measured according to the method described in AOAC (1984).

Preparation of acetone dry powder of cocoa beans

The unfermented and fermented cocoa bean samples were immediately frozen (−20°C) and freeze-dried

(Labconco, 100 μHg vacuum, −5°C). The testa and germs were then removed. The dry cotyledons (moisture content < 3%) were then crushed before they were defatted with petroleum ether (b.p. 40–60°C) for 8 h in a Soxhlet apparatus. The partially defatted cotyledons were ground and re-extracted for another 8 h. Following that, purine alkaloids were also removed by extraction with chloroform for 8 h. The resultant defatted and purine-free alkaloid cotyledons were then sieved (300 μm mesh) to obtain a uniform particle size cotyledon powder.

Acetone dry powder of cocoa beans was prepared according to the modified method of Kirchhoff *et al.* (1989). In order to remove polyphenols, 50 g of the powders were treated with cold aqueous acetone (kept at −20°C overnight) containing 5 mM sodium ascorbate and 0.1% thioglycolic acid. The mixture was shaken vigorously for 30 s and then incubated at −20°C for 1 h with frequent shaking at 20 min intervals. The suspension was then centrifuged at 10 000*g* for 15 min at 4°C and the resulting pellet was re-extracted twice with 80% cold acetone, and four times with 70% cold aqueous acetone. The residual water was removed by dehydration with 100% acetone. The resultant acetone dry powder was stored at −20°C before analysis.

Extraction of vicilin-class globulins of cocoa beans

Vicilin-class globulins were extracted according to the method described by Voigt *et al.* (1993) with slight modification. Ten grammes of acetone dry powder was extracted with 1 litre of Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl, 2 mM EDTA, 5 mM sodium ascorbate and protease inhibitors (10 μM pepstatin A and 1 mM phenylmethyl sulphonylfluoride) to avoid proteolytic digestion during extraction. The mixture was stirred for 12 h at 5 ± 1°C and then centrifuged at 10 000*g* for 20 min at 4°C. The resulting pellet was re-extracted three times with the same buffer. The remaining supernatants were pooled and 10% (v/v) thiochloroacetic acid (TCA) was added to it. The mixture was then chilled in an ice bath for 45 min to precipitate the vicilin fractions. The TCA pellet was washed with ethanol–ether (1:1) and then with excess deionized water. The resultant pellet was dialysed against deionized water at 5 ± 1°C for 2 days before it was freeze-dried (Labconco, 100 μHg vacuum, −5°C).

Determination of proteolytic activity

Acetone dry powders prepared from unfermented and fermented cocoa beans were blended with chilled sodium phosphate (pH 6.5) for 10 min at 4°C. After blending, the solution was centrifuged at 10 000*g* for 30 min at 4°C. The supernatant was dialysed against the same buffer at 5 ± 1°C for 3 days. Casein (0.5%) was used as the proteolytic substrate. Proteolytic activity was measured as the difference of absorbance at 280 nm after 1 h and at 0 h incubation at 37°C.

Determination of protein

Protein was determined according to Lowry *et al.* (1951) and bovine serum albumin was used as the standard.

Polyacrylamide gel electrophoresis of vicilin-class globulins

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970) in vertical slab gels (15 cm × 2 mm) with 12.5% T and 4.0% C, and 8–10 μg of proteins were loaded into each well. The relative molecular weights of the vicilin-class globulins were estimated by comparing relative mobolity (R_f) values of standard markers against R_f values of protein samples. The marker proteins used were bovine albumin (66 kDa), egg albumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18 kDa) and lysozyme (14 kDa). They were all obtained from Sigma Chemicals. Electrophoresis was carried out at a constant current of 40 mA for stacking gels and 30 mA for separating gels. Gels were stained and destained with Coomassie brilliant blue (R-250) and acetic acid–methanol solution, respectively, until the backgrounds were clear. They were scanned on a Shimadzu CS-9000 Scanning Densitometer at 552 nm.

RESULTS AND DISCUSSION

Partial purification of vicilin-class globulins

Acetone dry powder (AcDP) free of polyphenols is a prerequisite for a reliable protection of proteins against denaturation by quinones during the extraction procedure (Forsyth *et al.*, 1958). Vicilin-class globulins (VCG) from AcDP of unfermented and fermented cocoa beans were extracted based on their solubility in salt buffer solution. The pattern of protein bands detected is shown in Fig. 1 (lane a). Four protein bands were visually observed; band I (47.1 kDa), band II (39.2 kDa) and band III (21.1 kDa), while band IV was a low molecular weight polypeptide. The two polypeptides with apparent molecular weights of 47.1 and 39.2 kDa were approximately similar to those obtained by Voigt *et al.* (1993). They found that the polypeptides of globulin fractions with apparent molecular weights of 47 and 31 kDa were vicilin-class globulin subunits, both shown to be important for the formation of cocoa-flavour precursors during fermentation of cocoa bean. The third band (21.1 kDa) obtained in our study was similar to that found in previous studies, which was an albumin fraction and thought to be an amylase inhibitor (Spencer & Hodge, 1991) or a trypsin inhibitor (Dodo & Furtek, 1994) (Fig. 1, lanes a–d; band III). The 21.1 kDa protein showed inhibitory activities toward proteinases, particularly serine proteinases such as trypsin, chymotrypsin and subtilisin (Dodo & Furtek, 1994). The polypeptide of 21.1 kDa was also found to be the predominant protein in cocoa beans and it has

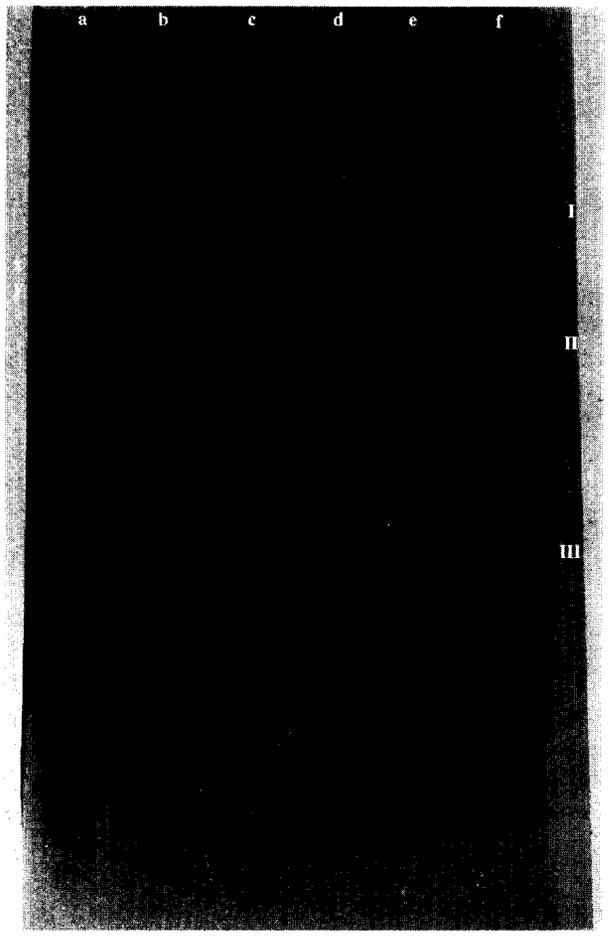

Fig. 1. Electrophoretic patterns of vicilin-class globulin and albumin fractions from cocoa beans during fermentation. (a) Unfermented bean; (b) VCG day 2 (48 h); (c) VCG day 3

accounted for 52% of total protein (Voigt *et al.*, 1993). The extraction procedure used to extract vicilin-class globulins in our study resulted in the presence of albumin as a major contaminant. Marcone *et al.* (1994) have also detected albumin in *Amaranthus hypochondriacus* seeds using 0.5 M NaCl, which is the same salt concentration as used in the present study. MacDonald *et al.* (1994) were able to separate the two albumin fractions from cocoa bean by ion-exchange chromatography (QAE-Sepharose) and chromatofocusing.

Effect of fermentation on vicilin-class globulin proteins

Fermentation is necessary for the development of flavour precursors in fermented cocoa beans. Cocoa flavour cannot be produced by the roasting of unfermented beans (Ziegleder & Biehl, 1988).

From SDS–PAGE (Fig. 1, lanes a–c; band I), it was found that a 47.1 kDa polypeptide was present in the fermentation at 0, 48 and 72 h and was absent at 96 and 144 h (Fig. 1, lanes d and e). Studies by Biehl *et al.* (1982) found that the same polypeptides disappeared at 72 h of fermentation. The difference could be due to the different fermentation techniques used. Our study used shallow wooden box fermentation (144 h fermentation, with turning at 48 and 96 h) which is currently practiced by Malaysian farmers, whereas Biehl *et al.* (1982) used fermentation-like incubation. The 46 and 46.5 kDa polypeptides (Fig. 1, lanes d and e; bands x and y) appeared at 96 and 144 h and were previously absent at 0, 48 and 72 h. Thus, from this observation, there is a possibility that the 47.1 kDa polypeptide had degraded to give the smaller-sized 46 and 46.5 kDa polypeptides; these bands cannot be from the 39.2 kDa polypeptide because their molecular weight is higher. The 39.2 kDa polypeptide was absent at the end of fermentation (Fig. 1, lane e; band II). This is in agreement with the previous studies by Voigt *et al.* (1993). They found the vicilin-class globulin (39.2 kDa) was completely degraded at the end of fermentation.

Table 1. Protein amounts of the vicilin-class globulin and an albumin at different stages of cocoa fermentation

Band	Relative molecular weight (kDa)	Protein concentration (μg protein)				
		0	2	3	4	6*
I	47.1	1.74	0.47	0.41	0.03	0.03
x	46.5	0.06	0.20	0.29	0.32	0.34
y	46.0	0.03	0.03	0.06	0.16	0.23
II	39.2	0.93	0.34	0.21	0.01	0.02
III	21.1	2.48	6.56	7.31	7.90	7.90

*Days of fermentation using shallow wooden boxes.

Table 1 shows the protein concentration of both vicilin-class globulins (bands I and II) and an albumin (band III) at different stages of fermentation. As fermentation progresses from 48 to 144 h, the protein concentrations of 47.1 and 39.2 kDa polypeptides decreased from 1.74 to 0.03 μg and from 0.93 to 0.02 μg, respectively (Table 1). The degradation of these two polypeptides was due to endoproteases which split the protein chains to form oligopeptides (Biehl & Voigt, 1994). On the other hand, the protein concentrations of bands x and y (apparent molecular weights of 46 and 46.5 kDa, respectively), which were thought to be the degradation products of the 47.1 kDa polypeptide, increased from 0.06 to 0.34 μg and from 0.03 to 0.23 μg at the end of fermentation, respectively (Table 1). The optimal level of flavour precursors was produced when the vicilin-class globulin was completely degraded by endoproteases during fermentation (Voigt *et al.*, 1993). Therefore, it could be postulated that the increment of protein bands at the end of fermentation could be due to the fact that vicilin-class globulin (47.1 kDa) did not degrade completely to specific oligopeptides. The action of aspartic endoproteases on vicilin-class globulin would produce specific hydrophobic oligopeptides (Biehl & Voigt, 1994). Biehl and Voigt (1994) found that flavour precursors such as hydrophilic oligopeptides and hydrophobic free amino acids (leucine, alanine, phenylalanine and tyrosine) were produced by the action of carboxypeptidases on the specific hydrophobic oligopeptides.

Figure 2 shows the changes of proteolytic activity and pH of the cotyledons of beans during the course of

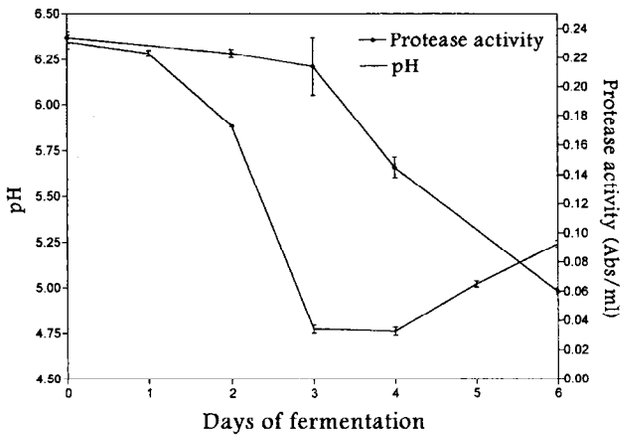

Fig. 2. pH and protease activity of cocoa beans during fermentation.

fermentation. There was a slight decrease in pH at 48 h followed by a sharper reduction at 72 h and then an increase until the end of fermentation. The unfermented cocoa beans (0 h) had a pH of 3.5 and 6.3 in the pulp and cotyledon beans, respectively. During the first 24–48 h of fermentation, ethanol and acids (acetic, lactic, succinic, malic and tartaric) develop in the pulp through sugar degradation by micro-organisms (Jinap, 1994). The acetic acid penetrates into the cotyledon, along with ethanol and the heat produced during fermentation, and causes bean death. Enzymatic reactions take place in the cotyledons as soon as bean death occurs. Our study indicates that as proteolytic activity decreased (Fig. 2), the intensity of vicilin-class globulin bands was reduced (Fig. 1, lanes b and c; bands I and II); however, at 96–144 h of fermentation, the bands are still present (Fig. 1, lanes d and e; bands x and y). The protein concentration of band III (21.1 kDa) increased during fermentation (0–144 h) from 2.48 to 7.00 μg (Table 1). The increase in albumin was due to the proteolysis of complex protein during fermentation (Zak & Keeney, 1976*b*). The 21.1 kDa protein did not degrade during fermentation (Fig. 1, lanes b–e; band III) which could be due its inhibitory behaviour towards proteases. At 72 h, when the pH was in the vicinity of 4.6, the proteolytic activity was higher (Fig. 2). This may be due to the aspartic endoprotease activity which has a pH optimum at pH 4.5. At the end of fermentation, when pH was approximately 5.8, the activity of carboxypeptidase was optimum. According to Voigt *et al.* (1994*a*), the optimum levels of hydrophobic oligopeptides were produced by the action of aspartic endoproteases on vicilin-class globulins at pH 4.5, while the formation of hydrophilic oligopeptides and hydrophobic free amino acids was caused by the action of carboxypeptidases on hydrophobic oligopeptides at pH 5.8.

In order to understand the behaviour of these two enzymes and aromas produced from roasted polypeptides, studies on enzyme characteristics and factors related to changes during cocoa fermentation should be carried out.

CONCLUSION

The study found that the vicilin-class globulin fraction (47.1 kDa) was not completely lost, in contrast to the 39.2 kDa polypeptide, which was completely degraded by the final day of fermentation. Two types of polypeptides with apparent molecular weights of 46 and 46.5 kDa appearing at 96 and 144 h of fermentation, and which were previously absent at 0, 48 and 72 h, could be the result of the degradation of 47.1 kDa polypeptides. Ninety-eight percent of the protein present in the 47.1 and 39.2 kDa proteins was lost at the end of fermentation. On the other hand, 82 and 87% of the protein present in the 46.5 and 46 kDa fractions had increased at the end of fermentation. The 21.1 kDa fraction could be an albumin fraction and was not degraded during fermentation.

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REFERENCES

AOAC (1984). *Official Methods of Analysis*, 14th edn. Association of Official Analytical Chemists, Washington, DC.

Biehl, B., Brunner, E., Passern, D., Quesnel, V. C. & Adomako, D. (1985). Acidification, proteolysis and flavour potential in fermenting cocoa beans. *J. Sci. Food Agric.*, **36**, 583–598.

Biehl, B. & Voigt, J. (1994). Biochemical approach to raw cocoa quality improvement: Comparison of seed proteins and proteases in their ability to produce cocoa aroma precursors. Paper presented at the Malaysian International Cocoa Conference, 20–21 October 1994, Kuala Lumpur, Malaysia.

Biehl, B., Wewetzer, C. & Passern, D. (1982). Vacuolar (storage) protein of cocoa seeds and their degradation during germination and fermentation. *J. Sci. Food Agric.*, **33**, 1291–1304.

Dodo, H. W. & Furtek, D. B. (1994). Cloning and sequencing of a gene encoding a 21 kDa trypsin inhibitor from *Theobroma cacao* L. *Cafe Cocoa*, **38**, 113–117.

Forsyth, W. G. C., Quesnel, V. C. & Roberts, J. B. (1958). The interaction of polyphenols and proteins during cocoa curing. *J. Sci. Food Agric.*, **9**, 181–184.

Jinap, S. (1994). Organic acids in cocoa beans—a review. *ASEAN Food J.*, **9**, 3–12.

Kirchhoff, P. M., Biehl, B. & Crone, G. (1989). Peculiarity of the accumulation of free amino acids during cocoa fermentation. *Food Chem.*, **31**, 295–311.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **277**, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MacDonald, H. M., Masters, K. E. & Pettipher, G. L. (1994). Partial purification of cocoa seeds proteins and studies into

the degradation of cocoa storage protein. *Cafe Cocoa*, **38**, 119–123.

Marcone, M. F., Niekamp, F. K., Maguer, M. L. & Yada, R. Y. (1994). Purification and characterization of the physicochemical properties of the albumin fraction from the seeds of *Amaranthus hypochondriacus*. *Food Chem.*, **51**, 287–294.

Spencer, M. E. & Hodge, R. (1991). Cloning and sequencing of the cDNA encoding the major albumin of *Theobroma cacao*. Identification of the protein as a member of the Kunitz protease inhibitor family. *Planta*, **183**, 528–535.

Spencer, M. E. & Hodge, R. (1992). Cloning and sequencing of a cDNA encoding the major storage protein of *Theobroma cacao*. Identification of the protein as members of the vicilin class of storage protein. *Planta*, **186**, 567–576.

Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Gaim Marsoner, G. & Hugi, A. (1994). In-vitro formation of cocoa-specific aroma precursors: aroma-related peptides generated from cocoa-seed protein by co-operation of an aspartic endoprotease and a carboxypeptidase. *Food Chem.*, **49**, 173–180.

Voigt, J., Biehl, B. & Kamaruddin, S. (1993). The major seed protein of *Theobroma cacao* L. *Food Chem.*, **47**, 145–147.

Voigt, J., Wrann, D., Heinrichs, H. & Biehl, B. (1994). The proteolytic formation of essential cocoa-specific aromaprecursors depends on particular chemical structures of the vicilin-class globulin of the cocoa seeds lacking in the globular storage proteins of coconuts, hazelnuts and sunflower seeds. *Food Chem.*, **51**, 197–205.

Zak, D. L. & Keeney, P. G. (1976). Extraction and fractionation of cocoa proteins as applied to several varieties of cocoa beans. *J. Agric. Food Chem.*, **24**, 479–483.

Zak, D. K. & Keeney, P. G. (1976). Changes in cocoa proteins during ripening of fruit, fermentation, and further processing of cocoa beans. *J. Agric. Food Chem.*, **24**, 483–486.

Ziegleder, G. & Biehl, B. (1988). Analysis of cocoa flavour components and flavour precursors. In *Methods in Plant Analysis*, Vol. 8 (new series), eds H. Linskens & J. F. Jackson. Springer, New York, pp. 321–393.