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Cocoa-specific aroma precursors are generated by proteolytic digestion of the vicilin-like globulin of cocoa seeds

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The proteolytic formation of the cocoa-specific aroma precursors was investigated *in vitro* using protein substrates and proteases purified from ungerminated cocoa seeds. An aspartic endoprotease and a carboxypeptidase present in ungerminated cocoa seeds were found to be required for this process. Cocoa-specific aroma precursors were obtained by proteolytic digestion of the vicilin-like globulin but not by proteolysis of the albumin of cocoa seeds.

INTRODUCTION

Fermentation of cocoa seeds is required to obtain cocoa-specific aroma upon roasting (Rohan, 1964). Therefore, essential precursors of the specific aroma components are generated during the fermentation process. Mohr and co-workers have extracted, fractionated and partially characterized the cocoa-specific aroma precursors from fermented cocoa seeds (Mohr *et al.*, 1971; 1976). These authors have reported that typical cocoa aroma was obtained when a peptide fraction isolated from fermented cocoa seeds was roasted in the presence of free amino acids and reducing sugars (Mohr *et al.*, 1976). These findings indicate that the cocoa-specific aroma precursors are formed during fermentation by proteolytic processes (Ziegleder & Biehl, 1988). Indeed, both the proteolysis of seed proteins and the formation of aroma precursors are strongly dependent on the degree and the time course of nib acidification during the fermentation process (Biehl *et al.*, 1982; 1985). Fermentations with moderate nib acidification (pH 5·0–5·5) result in raw cocoa batches with considerably higher aroma potentials than fermentations at pH 4·0–4·5 (Biehl *et al.* 1985)., The findings that there is a correlation between formation of cocoa-specific aroma precursors, proteolysis of seed proteins and the degree and the time course of acidification during the fermentation process, have led to the development of a procedure (post-harvest storage of cocoa pods) which has improved raw cocoa quality in Malaysia (Biehl *et al.*, 1989; Meyer *et al.*, 1989).

Ungerminated cocoa seeds contain a single endoprotease, i.e. an aspartic protease with maximal activity around pH 3·5 (Passern, 1979; Biehl & Passern, 1982; Biehl *et al.*, 1993). This enzyme should, therefore, be involved in the generation of the cocoa-specific aroma precursors. However, strong acidification of the nib during fermentation (pH 4·0–4·5) causes a low aroma potential, although the endoprotease is more active at even lower pH-values. On the other hand, accumulation of hydrophobic free amino acids has been found during fermentation or fermentation-like incubations of cocoa seeds (Kirchhoff *et al.*, 1989*a*, *b*). During fermentation-like seed incubations at pH 5·5, considerably higher amounts of amino acids were liberated than at pH 4·5 (Kirchhoff *et al.*, 1989*b*) indicating that an exopeptidase is also involved in the generation of cocoa-specific aroma precursors. To distinguish which enzymes are involved in this process, we have started to investigate the proteolytic formation of cocoa-specific aroma precursors *in vitro* using polyphenol-free acetone-dry powder prepared from unfermented cocoa seeds (Voigt *et al.*, 1994): By incubation of acetone-dry powder (AcDP) at pH 5·2, we obtained mixtures of hydrophilic peptides and predominantly hydrophobic free amino acids which, on roasting in the presence of reducing sugars and deodorized cocoa butter, unequivocally revealed typical cocoa aroma. No cocoa-specific aroma precursors were, however, generated when autolysis of AcDP was performed at pH 3·5. The hydrophobic peptides formed under these conditions could be transformed to mixtures of hydrophilic peptides and predominantly hydrophobic free amino acids by post-treatment with commercial carboxpeptidase. Again, these mixtures of hydrophilic peptides and hydrophobic free amino acids were shown to contain cocoa-specific aroma precursors. By this experimental approach, we were able to show that the cocoa-specific

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aroma precursors are generated from seed proteins by cooperation of an aspartic endoprotease and a carboxypeptidase (Voigt *et al.*, 1994).

Fermentation-like incubations of intact cocoa seeds under controlled laboratory conditions have revealed that at pH 4·0–4·5, an unspecific proteolysis of all the seed proteins takes place, whereas at pH 5·0–5·5 distinct vacuolar storage proteins are selectively degraded (Biehl *et al.*, 1982). The polypeptides which are selectively degraded during seed incubations at pH 5·0–5·5 have been shown recently to be the polypeptide subunits of a vicilin-type globulin (Spencer & Hodge, 1992; Voigt *et al.*, 1994) indicating that the cocoa-specific aroma precursors might be derived from vicilin-like globulin of the cocoa seeds. This assumption was corroborated by *in vitro* studies of the proteolytic formation of these particular aroma precursors using purified substrate proteins and proteases as shown in the present communication.

MATERIALS AND METHODS

Materials

Cocoa seeds were from ripe, genetically undefined pods harvested at the Cocoa and Coconut Division of the Malaysian Agricultural Research and Development Institute (MARDI, Hilir Perak, Malaysia), Unfermented seeds were taken from the pods immediately after arrival (4–5 days after harvesting), shock-frozen in liquid nitrogen after removal of testae and radiculae and freeze-dried.

Extraction of fat

The dry cotyledons were crushed and portions of 10 g each were extracted repeatedly in a Soxhlet apparatus with 500 ml of petroleum ether (bp 40–70 °C). After solvent evaporation, the material was powdered and extracted again for 8 h in the same manner. Finally, purine alkaloids were partially extracted with chloroform for 8 h in a Soxhlet apparatus.

Preparation of AcDP

AcDP of cocoa seeds was prepared essentially as recently described (Kirchhoff *et al.*, 1989*a*). To remove the polyphenols, the defatted seed powders were extracted three times with 80% (v/v) aqueous acetone containing 5 mM sodium ascorbate and subsequently with 70% (v/v) aqueous acetone. The suspensions (200 ml aqueous acetone per 10 g seed powder) were stirred for 1 h at 4°C and the extracts removed by centrifugation (15 min at 13000 × *g*). After the final extraction step, efficiency of polyphenol extraction was checked by heating an aliquot of the acetone dry powder with 5 M HCl (red colour indicates the presence of residual polyphenols). After complete extraction of polyphenols, residual water was removed by extraction with 100% acetone. After final centrifugation, the sediment was evaporated under reduced pressure to remove the solvent. The acetone dry powder (AcDP) was stored at –20°C.

Fractionation of seed proteins

Seed proteins were extracted from polyphenol-free AcDP as recently described (Voigt *et al.*, 1993*a*): AcDP (50 g) was first extracted with 5 litres of a low-salt buffer containing 5 mM sodium ascorbate, 2 mM EDTA and 10 mM Tris-HCl (pH 7·5) to obtain the albumin fraction. The suspension was stirred for 1 h at 4°C and subsequently centrifuged for 20 min at 20,000 × *g* and 4°C in the Sorvall GSA rotor (Du Pont de Nemours GmbH, Bad Homburg, Germany). Extraction was repeated three times to minimize a carry over of proteins soluble under low-salt conditions to the globulin fraction. Only the first two extracts were combined and stored at −70°C until use. Subsequently, the pretreated AcDP was extracted with 5 litres of 0·5 M NaCl containing 5 mM sodium ascorbate, 2 mM EDTA and 20 mM Tris-HCl (pH 7·5) to obtain the globulin fraction. The suspension was stirred at 4°C for 1 h and centrifuged for 20 min at 20000 × *g* and 4°C in the Sorvall GSA rotor. The extraction was repeated and the supernatants combined. When the high-salt extract was dialysed against distilled water and subsequently against 20 mM sodium acetate (pH 5·0), a precipitate was formed which was found to consist of almost pure vicilin-like globulin (Fig. 1, lane d; cf. Pettipher, 1990).

The low-salt extract was fractionated by anion exchange chromatography using a Whatman DE-52 column (50 mm × 250 mm) equilibrated with 10 mM sodium phosphate (pH 7·5). The low-salt extract was directly applied to the column. After washing with 500 ml equilibration buffer containing 5 mM sodium ascorbate, the column was eluted with a linear gradient of 100–400 mM NaCl in equilibration buffer containing 5 mM sodium ascorbate (2 litres each). Fractions of 20 ml were collected and measured for absorbance at 280 nm and the activities of aspartic endoprotease, carboxypeptidase and leucine-*p*-nitroanilide cleaving enzyme. Furthermore, the eluate fractions were analyzed for the presence of the 19 kDa albumin by SDS-PAGE according to Laemmli (1970). Peak fractions were combined, concentrated by treatment with Aquacide II (Calbiochem-Behring, Marburg, Germany), dialyzed against 10 mM sodium phosphate (pH 7·5) and stored at −20°C.

Enzyme assays

Aspartic endoprotease

Aspartic endoprotease activity was measured in McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to pH 3·5 by addition of citric acid). The 1-ml reaction mixtures containing 9 mg of bovine serum albumin as substrate were incubated at 45°C for 1 h. The reaction was

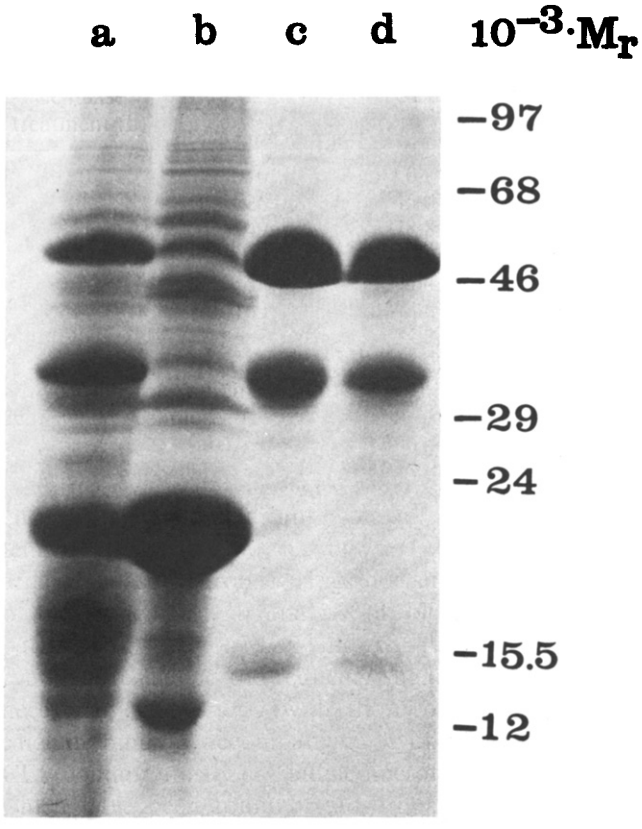

Fig. 1. SDS-PAGE profiles of the protein fractions prepared from ripe cocoa seeds. The protein fractions were obtained by solubility fractionation starting from polyphenol-free acetone-dry powder as described in the 'Materials and Methods' section, and 60 μg of each fraction were subjected to SDS-PAGE according to Laemmli (1970). The gels were stained with Coomassie brillant blue. The molecular weights (M_r) of protein standards (phosphorylase b = 97000; bovine serum albumin = 68000; ovalbumin = 46000; carbonic anhydrase = 29000; trypsin inhibitor 24000; haemoglobin = 15500; cytochrome c = 12.300) are indicated by the numbers on the right. (a) Total seed proteins; (b) crude albumin; (c) crude globulin; and (d) globulin purified by precipitation at low salt concentration and acidic conditions.

stopped by addition of 0·2 ml of trichloroacetic acid (25%, w/v). The precipitated protein was removed by centrifugation at 10000 × *g* for 15 min. Subsequently, proteolysis was measured colorimetrically by the trinitro-benzenesulfonic acid method as described by Shutov *et al.*, (1982). Each sample was analysed for protease activity both in the presence and absence of 10 μg pepstatin A (Sigma Chemie, Deisenhofen, Germany). One unit of aspartic endoprotease is the pepstatin sensitive activity which releases 1 μmol NH_2-groups per minute at pH 3·5 (0·2 M McIlvaine buffer) and 45°C.

Carboxypeptidase activity

Samples were first preincubated in the presence of 10 μg/ml pepstatin A (Sigma Chemie, Deisenhofen, Germany) for 1 h in an ice bath to inhibit the aspartic endoprotease (Biehl *et al.*, 1993). Aliquots were then added to 0·9 ml of McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to pH 5·8 by addition of citric acid) containing 5 mM Z-Phe-Leu (Sigma Chemie, Deisenhofen, Germany) added from a 125 mM stock solution in methanol. The reaction mixtures were incubated at 45°C. After 3 h, the reaction was stopped by addition of 0·2 ml of 25% (w/v) trichloroacetic acid and the precipitated protein removed by centrifugation at 10000 × *g* for 15 min. Finally, the released leucine was determined colorimetrically by the trinitrobenzenesulfonic acid method as described by Shutov *et al.*, (1982). One unit of carboxpeptidase is the activity which releases 1 μmol leucine per min at pH 5·8 and 45°C.

Leucine-p-nitroanilide cleaving activity

Samples were preincubated in the presence of 10 μg/ml pepstatin A (Sigma Chemie, Deisenhofen, Germany) for 1 h in an ice bath to inhibit contaminating aspartic endoprotease. Aliquots (0·2 ml) of the preincubated samples were mixed with 0·2 ml of 2 mM L-leucine-*p*-nitroanilide (Sigma Chemie, Deisenhofen, Germany) and 5·6 ml of McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to pH 6·8 by addition of citric acid). The reaction mixtures were incubated at 45°C for 2h. Finally, the hydrolytic liberation of *p*-nitroaniline was determined photometrically at 400 nm (Passern, 1979; Biehl *et al.*, 1993). One unit is the activity which cleaves 1 μmol leucine-*p*-nitroanilide per min under the conditions described above.

Determination of protein

Protein concentrations were determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Electrophoresis

SDS-PAGE was performed on gel slabs (140 mm × 140 mm × 1·5 mm) according to Laemmli (1970). The protein fractions to be analyzed were subjected to TCA precipitation and the precipitates repeatedly washed with distilled water and redissolved in urea-SDS buffer containing 8 M urea, 2% (w/v) SDS, 2 mM EDTA, 20 mM Tris-HCl (pH 7·5) and 200 mM 2-mercapto-ethanol (Voigt, 1985). After addition of 0·25 volumes of sample buffer according to Laemmli (1970) containing 0·005% (w/v) bromophenol as tracking dye, the samples were subjected to SDS-PAGE analysis. Gels were stained for protein with Coomassie brillant blue R250.

Digestion of purified seed proteins with cocoa aspartic endoprotease

Purified substrate proteins (5 g) were dissolved or suspended in 2 litres of distilled water. After addition of 100 mg of partially purified aspartic endoprotease (0·5 units/mg), the solutions were adjusted to either pH 5·2 or pH 3·5 by addition of acetic acid and incubated at 50°C. Another 100 mg of partially purified aspartic endoprotease were added after 3 h. After 16 h, methanol was added to a final concentration of 70 % (v/v). The suspensions were stirred at room temperature for 1 h and centrifuged at 20000 × *g* for 30 min. The super-

natants were collected and the methanol removed under reduced pressure at 40°C by means of a rotary evaporator. Finally, the aqueous solutions were freeze-dried.

Digestions with carboxypeptidase

Peptide mixtures (2–3 g) obtained by proteolysis of purified protein substrates with aspartic endoprotease were dissolved in 2 litres distilled water and the solutions adjusted to pH 5·8. After addition of 300 units of partially purified carboxypeptidase from cocoa seeds the solutions were incubated at 45°C in a shaking water bath. After 3 h, another 300 units units of carboxypeptidase were added. Incubations were stopped after 16 h. The resulting mixtures of peptides and free amino acids were freeze-dried.

Sensory evaluation

Proteolysis products (0·75 g) were mixed with 0·25 g glucose, 0·75 g fructose and 0·3 g water. After addition of 8·25 g deodorised cocoa butter, the mixtures were formulated either with mortar and pestle or by ultrasonication for 30 s using a Branson Sonifier B12 at maximal power. Finally, the samples were filled into glass Petri dishes as thin layers (2–3 mm) and roasted for 10–15 min in an oven preheated to 120°C. The generated aromas were evaluated by sniffing analysis. Evaluations of the roasting aromas were performed by a panel of 10 test persons. The sensory evaluation of the aromas obtained was restricted to a qualitative identification of aroma notes. The proportion of test persons recognizing cocoa aroma was found to be the only reliable value to evaluate the roasting aromas. Each aroma analysis was repeated at least three times with different preparations of the same type.

HPLC analyses of peptides

Peptide mixtures were analysed by reversed-phase HPLC as recently described (Voigt *et al.*, 1994) using the HPLC system Gold (Beckman Instruments, San Ramon, CA, USA) equipped with an Ultrasphere ODS 5μm column (4·6 mm x 250 mm). Elution of the peptides was performed at 30°C and a flow rate of 1 ml/min with 0·1% (v/v) aqueous trifluoroacetic acid (7 min) and subsequently with a linear gradient from 0 to 50% (v/v) acetonitrile containing 0·1% (v/v) trifluoroacetic acid (Bennett *et al.*, 1980; Mahoney & Hermodson 1980). The eluting peptides were monitored by measuring the absorbance of the effluents at 210 nm. No residual caffeine or theobromine were present in the peptide mixture as revealed by HPLC analysis of methanol extracts prepared from non incubated mixtures of proteases and protein substrates.

Amino acid analyses

The amino acids were converted into the *o*-phthalaldehyde (OPA) derivatives, separated by reversed-phase HPLC using a Shandon Hypersil ODS 5 (240 mm x 4·6 mm) column and a Shandon Hypersil ODS 10 (20 mm x 4·6 mm) precolumn as recently described (Kirchhoff *et al.*, 1989*a*). The effluents were monitored fluorometrically with a Hitachi model F-3000 spectrofluorometer (excitation at 334 nm; emission measured at 425 nm).

RESULTS

As recently reported, cocoa-specific aroma precursors can be produced *in vitro* (Voigt *et al.*, 1994)

- either by autolysis at pH 5·2 of AcDP prepared from unfermented ripe cocoa seeds,
- or by autolysis of AcDP at pH 3·5 and subsequent digestion of the resulting hydrophobic peptides with commercial carboxypeptidase A from porcine pancreas.

These findings indicate that the cocoa-specific aroma precursors are generated by cooperative digestion of seed proteins by an (aspartic) endoprotease and a carboxypeptidase present in ungerminated cocoa seeds. Subsequently, we have investigated from which seed protein(s) the cocoa-specific aroma precursors are derived.

To isolate the protein substrates and proteases present in ungerminated cocoa seeds, we have first extracted the AcDP under low-salt conditions to obtain the albumin fraction (Fig. 1, lane b). Then the globulin fraction was extracted by treatment with 0·5 M NaCl. This crude globulin fraction still contained slight contaminations of albumin(s) (Fig. 1, lane c). To purify the globulin, the high-salt extract was dialysed against distilled water and 10 mM sodium acetate (pH 5·0). The precipitate formed (= purified globulin) was collected by centrifugation. Analysis of the purified globulin fraction by SDS-PAGE (Fig. 1, lane d) revealed that

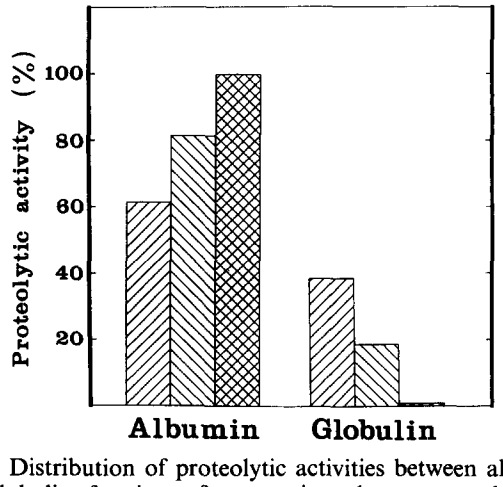

Fig. 2. Distribution of proteolytic activities between albumin and globulin fraction of ungerminated cocoa seeds. The albumin and the globulin fractions were prepared and analysed for protease activities as described in the 'Materials and Methods'section. Values are expressed as percentage of total enzyme units found in both fractions. ▨, Aspartic endoprotease; ▧, leucine-*p*-nitroanilide peptidase; ▩, carboxypeptidase.

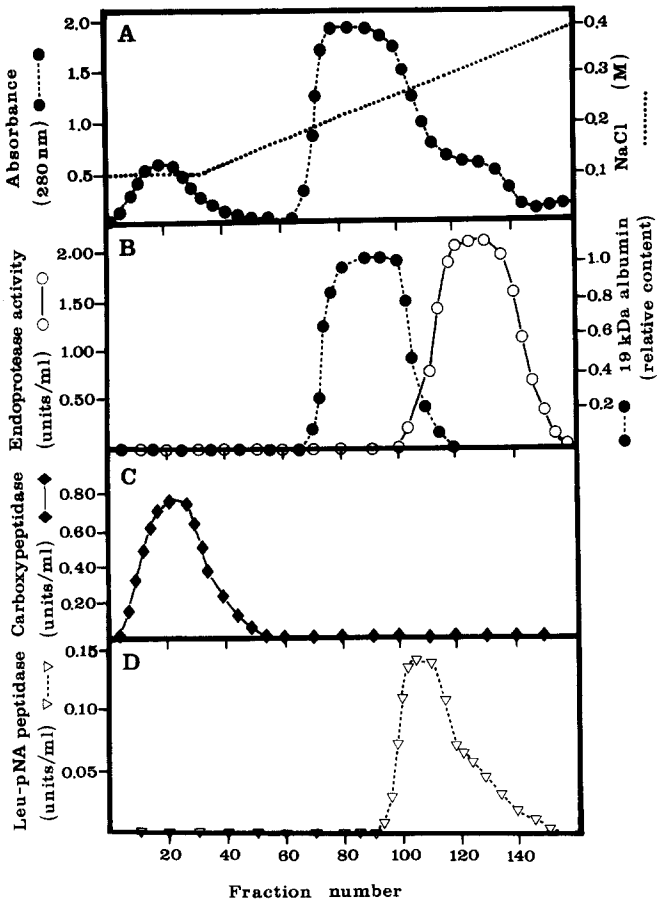

Fig. 3. Partial purification of proteolytic activities and the 19 kDa albumin by DEAE-cellulose chromatography of the crude albumin extract from ungerminated cocoa seeds. The crude albumin extract obtained from 50 g acetone-dry powder was directly applied to a Whatman DE-52 column (50 mm × 250 mm) and fractionated by a linear gradient of 100–400 mM NaCl as described in the 'Materials and Methods' section. The eluate fractions (20 ml) were analysed for absorbance at 280 nm (A); endoprotease activity (B); carboxypeptidase activity (C); and leucine-*p*-nitroanilide (Leu-pNA) peptidase activity (D). The distribution (relative content) of the 19 kDa albumin (B) was determined by SDS-PAGE analysis of the eluate fractions. The endoprotease activity (B) was found to be sensitive to pepstatin A.

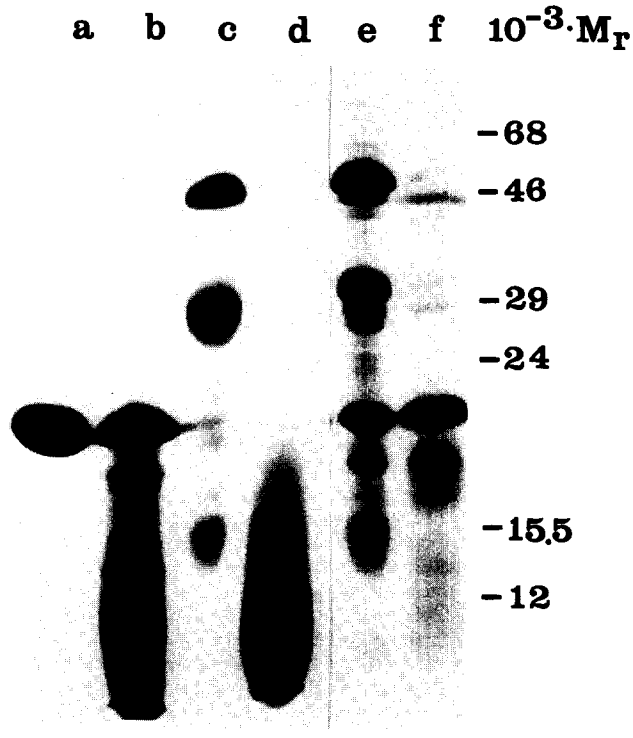

Fig. 4. SDS-PAGE analysis of the products formed during incubation of the albumin and the globulin from cocoa seeds in the presence and absence of aspartic endoprotease. Albumin and globulin, respectively, prepared from acetone-dry powder of ungerminated cocoa seeds were incubated at pH 3·5 and 50°C in the absence or presence of aspartic endoprotease (20 μg per mg protein substrate) as described in the 'Materials and Methods'section. After 16 h, aliquots of the reaction mixtures corresponding to 70 μg protein were subjected to SDS-PAGE analysis according to Laemmli (1970). The gels were stained with Coomassie brillant blue. The molecular weights of protein standards (M_r) are indicated by numbers on the right. 19 kDa Albumin incubated in the absence (a), or the presence of aspartic endoprotease (b); globulin incubated in the absence (c), or the presence of aspartic endoprotease (d); mixture of albumin and globulin incubated in the absence (e), or the presence of aspartic endoprotease (f).

it only contained the polypeptide subunits (47 kDa, 31 kDa, 15·5 kDa and 14·5 kDa) of the vicilin-like globulin of the cocoa seeds (Spencer & Hodge, 1992; Voigt *et al.*, 1993).

Analysis of the protease activities of ungerminated cocoa seeds revealed the presence of an aspartic endoprotease, a carboxypeptidase activity and a leucine-*p*-nitroanilide-cleaving enzyme (Biehl & Passern, 1982; Biehl *et al.*, 1991, 1993). Therefore, we have studied the distribution of these proteolytic activities in the crude albumin and globulin fractions prepared from AcDP. As shown in Fig. 2, the predominant proportions of all these enzyme activities were found in the albumin fraction. No proteolytic activity was found in the globulin fraction purified by precipitation under low-salt conditions (dialysis against distilled water and subsequently against 10 mM sodium acetate, pH 5·0).

To separate the predominant albumin from the different protease activities, the crude albumin fraction was subjected to DEAE-cellulose chromatography. As shown in Fig. 3, the carboxypeptidase activity, the 19 kDa albumin and the aspartic endoprotease were sufficiently separated from each other under the chromatographic conditions used. The carboxypeptidase was eluted at the beginning of the gradient (100 mm NaCl), the aspartic endoprotease near the end of the gradient (330–390 mm NaCl). The 19 kDa albumin appeared as a broad peak between 250 and 350 mm NaCl. The leucine-p-nitroanilide cleaving enzyme was eluted between 300 and 360 mm NaCl. Therefore, both the 19 kDa albumin and the aspartic endoprotease were more or less contaminated by the leucine-p-nitroanilide cleaving enzyme.

No degradation of the purified albumin and globulin, respectively, was observed when these proteins were incubated without addition of aspartic endoprotease (Fig. 4, lanes a and c). Both the globulin and the 19 kDa albumin were degraded by the partially purified aspartic endoprotease (Fig. 4, lanes b and d). However,

Table 1. Cocoa-specific aroma potentials of proteolysis products generated *in vitro* from the albumin and the globulin fractions of cocoa seeds by the cooperative action of aspartic endoprotease and carboxypeptidase from cocoa seeds[a]

Protein substrate	Protease[b]	Cocoa-specific aroma potential[c]
I Globulin	(1) Aspartic endoprotease (pH 5·2)	0/10
	(2) —	(0)
II Globulin	(1) Aspartic endoprotease (pH 3·5)	0/10
	(2) —	(0)
III Globulin	(1) Aspartic endoprotease (pH 5·2)	5/10
	(2) Carboxypeptidase (pH 5·8)	(50)
IV Globulin	(1) Aspartic endoprotease (pH 3·5)	6/10
	(2) Carboxypeptidase (pH 5·8)	(60)
V Albumin	(1) Aspartic endoprotease (pH 5·2)	0/10
	(2) Carboxypeptidase (pH 5·8)	(0)

[a] The albumin and the globulin fraction were prepared from the acetone-dry powder of ungerminated cocoa seeds, subjected to proteolysis with endo- and exopeptidases from cocoa seeds, and the resulting protelysis products analysed for cocoa-specific aroma potential.
[b] The protein substrates were digested for 16 h at 50°C with aspartic endoprotease from cocoa seeds at pH 5·2 or pH 3·5 (incubation 1). Where indicated, the methanol-soluble peptides obtained were adjusted to pH 5·8 and treated with carboxypeptidase from cocoa seeds as described in the 'Material and Methods' section (incubation 2).
[c] The freeze-dried proteolysis products were formulated and roasted in the presence of reducing sugars as described in the 'Material and Methods' section. Sensory evalution of the obtained roasting aromas was performed by sniffing analysis. Values are given as the number of test persons who have recognized cocoa or chocolate aroma, respectively, versus the number of test persons. Values in parentheses indicate the percentage of test persons who have recognized cocoa and/or chocolate aroma

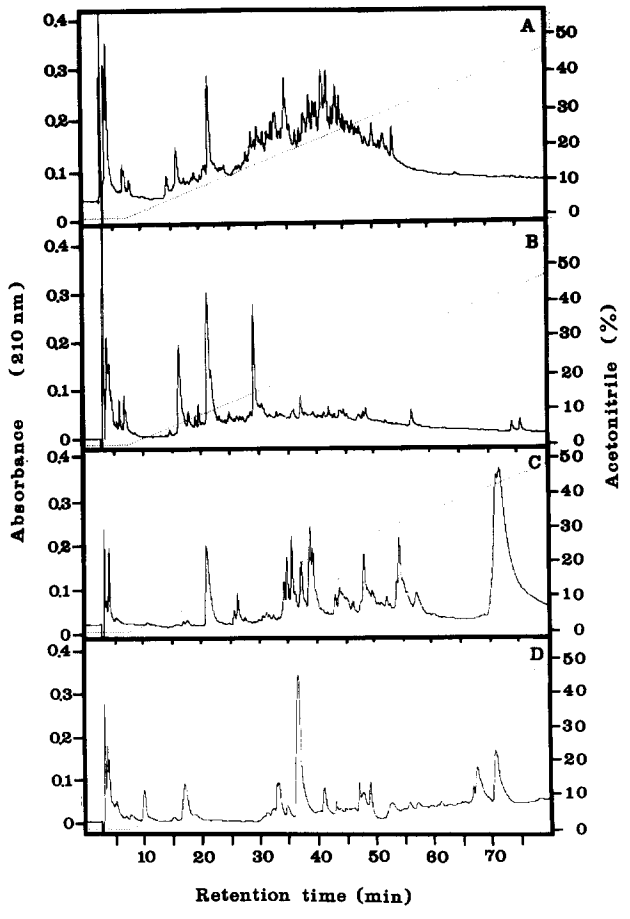

Fig. 5. Reversed phase HPLC analysis of peptide mixtures obtained by proteolytic digestion of the globulin (A, B), and the 19 kDa albumin (C, D). The globulin and the 19 kDa albumin from cocoa seeds were digested with aspartic endoprotease without (A, C), or with post-treatment with carboxypeptidase from ungerminated cocoa seeds (B, D), and the resulting peptide mixtures analysed by reversed-phase HPLC as described in the 'Materials and Methods'section. Globulin digested with aspartic endoprotease (at pH 3·5) without (A), or with post-treatment (at pH 5·8) with carboxypeptidase from ungerminated cocoa seeds (B); 19 kDa albumin digested with aspartic endoprotease (at pH 3·5) without (C), or with post-treatment (at pH 5·8) with carboxypeptidase from cocoa seeds (D).

the globulin was found to be more susceptible to proteolytic digestion by this particular enzyme than the 19 kDa albumin. Under the same conditions, where a complete degradation of the globulin was observed (Fig. 4, lane d), a considerable proportion of the 19 kDa albumin was apparently not cleaved by the endoprotease (Fig. 4, lane b). This differential proteolysis was obvious when, in another experiment, a mixture of albumin and globulin was incubated in the presence of aspartic endoprotease (Fig. 4, lane f). At temperatures below 40°C and pH-values above pH 5·0, no degradation of the 19 kDa albumin by the aspartic endoprotease was observed (data not shown) whereas the globulin was efficiently attacked under these conditions.

The albumin and the vicilin-like globulin purified from ripe ungerminated cocoa seeds were subjected to proteolytic digestion by aspartic endoprotease in a preparative scale with or without post-treatment with carboxypeptidase. The obtained proteolysis products were roasted in the presence of reducing sugars and deodorized cocoa butter and the resulting aromas sensorically evaluated by a panel of 10 people. No cocoa or chocolate aroma was detected by any test person in the case of peptide mixtures obtained by proteolysis of purified globulin with partially purified aspartic endoprotease only (Table 1, I and II). However, when these peptides were post-treated with carboxypeptidase, five to six out of the ten panellists recognized typical cocoa aroma upon roasting in the presence of reducing sugars (Table 1, III and IV). The same results were obtained when digestion of the globulin by aspartic endoprotease was performed at pH 5·2 or pH 3·5 (Table 1, III and IV). However, post-treatments with the carboxypeptidase from cocoa seeds had to be performed at pH > 5·0. Proteolysis products of the albumin with partially purified aspartic endoprotease and carboxypeptidase from cocoa seeds did not reveal cocoa or chocolate aroma upon roasting in the presence of reducing sugars and deodorized cocoa butter (Table 1, V).

The patterns of oligopeptides and free amino acids in the different proteolysis products were comparatively analysed (Fig. 5, Table 2). Reversed-phase HPLC of the oligopeptides formed by digestion of the vicilin-like globulin and the albumin, respectively, with the aspartic endoprotease, revealed different, complex patterns of more or less hydrophobic components (Fig. 5A, C). These mixtures of hydrophobic peptides were transformed to more hydrophilic components by treatment with carboxypeptidase from cocoa seeds (Fig. 5B, D).

Table 2. Free amino acids present in the proteolysis products generated in vitro by degradation of the albumin or the globulin from cocoa seeds with aspartic endoprotease and carboxypeptidase from cocoa seeds[a]

Amino acid	Globulin				Albumin	
	Aspartic endoprotease pH 3·5		Aspartic endoprotease pH 5·2		Aspartic endoprotease pH 5·2	
	Minus CP	Plus CP	Minus CP	Plus CP	Minus CP	Plus CP
Asp	0·64	46·7	0·57	45·2	0·67	79·6
Glu	0·68	54·4	0·76	53·1	0·73	48·8
Asn	0·74	63·5	0·73	68·3	0·79	38·2
Ser	0·86	42·6	0·67	44·3	0·88	14·8
Gln + His	0·13	83·1	0·14	65·5	0·24	10·8
Gly	0·29	21·1	0·23	26·0	0·35	10·7
Thr	0·37	44·1	0·47	49·8	0·82	10·8
Arg	0·80	52·9	0·78	49·1	0·72	12·9
Ala	1·27	135·4	1·07	123·5	0·23	19·0
Tyr	0·49	68·7	1·06	70·4	0·07	12·5
Trp	0·09	16·3	0·08	15·7	0·07	8·2
Met	0·11	46·7	0·15	44·0	0·12	11·3
Val	0·74	114	0·75	93·8	0·42	9·95
Phe	1·27	183	2·15	135·5	0·97	22·5
Ile	0·70	65·1	0·87	56·7	0·41	9·65
Leu	0·99	180	1·73	124	0·91	31·2
Lys	0·08	0·10	0·09	0·10	0·07	0·11

[a] The albumin and the globulin, respectively, prepared from ripe, ungerminated cocoa seeds was digested with aspartic endoprotease from cocoa seeds as described in the 'Materials and Methods' section. Free amino acids present in the proteolysis products prior to (minus CP) and after post-treatment with carboxypeptidase from cocoa seeds (plus CP) were analysed by reversed-phase HPLC of the OPA derivatives (Kirchhoff *et al.*, 1989*a*). CP, carboxypeptidase from ungerminated ripe cocoa seeds. Values in nmol/mg dry weight.

In the case of the globulin, the very complex pattern observed after incubation with aspartic endoprotease (Fig. 5A) was altered considerably by post-treatment with carboxypeptidase. Only a few dominant peaks were observed (Fig. 5B). These findings indicate that a considerable proportion of the oligopeptides formed by cooperation of aspartic endoprotease and the carboxypeptidase are not separated by reversed-phase HPLC.

Very low amounts of free amino acids were found in the proteolysis products obtained by digestion of the albumin and the globulin, respectively, with the aspartic endoprotease only (Table 2). When the globulin peptides were treated with carboxypeptidase, hydrophobic amino acids—especially leucine, phenylalanine, alanine, valine and tyrosine—were preferentially released (Table 2). Globulin peptides generated by aspartic endoprotease at pH 5·2 and pH 3·5, respectively, revealed the same patterns of free amino acids after carboxypeptidase treatment (Table 2). Hydrophobic amino acids—especially leucine, phenylalanine and alanine—were also liberated by carboxypeptidase from the albumin peptides (Table 2). However, the predominant amino acids released from the albumin-derived oligopeptides by carboxypeptidase treatment were aspartic acid, glutamic acid and asparagine (Table 2).

DISCUSSION

In the past three decades, evidence has shown that essential precursors of the cocoa-specific aroma components are generated by proteolytic processes occuring during fermentation of cocoa seeds (Rohan, 1964; Mohr *et al.*, 1971, 1976; Biehl *et al.*, 1985; Ziegleder & Biehl, 1988). Incubations of cocoa seeds under aseptic conditions have revealed that a moderate acidification of the seeds (to pH 5·5–5·0), but not the presence of microorganisms is required for the formation of the typical aroma precursors (Biehl *et al.*, 1985). The conclusion that acid induced proteolysis of seed proteins occurs due to endogenous proteases (Biehl *et al.*, 1982, 1985), has been recently corroborated by the *in vitro* formation of cocoa-specific aroma precursors (Voigt *et al.* 1993*b*, 1994). Typical aroma precursors were generated during autolysis of AcDP from unfermented cocoa seeds at pH 5·2, but not at pH 3·5. Autolysis of AcDP at pH 3·5 revealed a complex mixture of hydrophobic oligopeptides. Cocoa-specific aroma precursors were obtained when these mixtures of hydrophobic oligopeptides were treated with carboxypeptidase A from porcine pancreas (Voigt *et al.*, 1993*b*, 1994). These findings indicated that the typical aroma precursors present in fermented cocoa seeds are generated from seed proteins by cooperation of an (aspartic) endoprotease and a carboxypeptidase (Voigt *et al.*, 1994). Furthermore, these findings lead to the identification of the seed protein from which the cocoa-specific aroma precursors are derived.

Cocoa seeds contain two major proteins: a 19 kDa albumin and a vicilin-like globulin (Spencer & Hodge, 1991, 1992; Tai *et al.*, 1991; McHenry & Fritz, 1992; Voigt *et al.*, 1993*d*). As shown in the present communi-

cation, cocoa-specific aroma precursors were obtained when the isolated globulin was successively degraded by the aspartic endoprotease and the carboxypeptidase partially purified from ungerminated cocoa seeds. No typical aroma precursors were obtained when the albumin fraction was subjected to to proteolysis by aspartic endoprotease and carboxypeptidase from cocoa seeds. Together with the earlier findings that, under fermentation conditions, where raw cocoas with high flavour potentials were obtained, when the polypeptide subunits of the globulin were selectively degraded (Biehl *et al.*, 1982; Voigt *et al.*, 1993*a*), these findings clearly show that the cocoa-specific aroma precursors are derived from the vicilin-like globulin of the cocoa seeds.

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