ELSEVIER

Food Chemistry 51 (1994) 7–14

0308-8146/94/$7.00

In vitro studies on the proteolytic formation of the characteristic aroma precursors of fermented cocoa seeds: The significance of endoprotease specificity

J. Voigt, G. Voigt, H. Heinrichs, D. Wrann & B. Biehl

Botanisches Institut, Technische Universität Braunschweig, Mendelssohnstraße 4, D-38092 Braunschweig, Germany

(Received 24 November 1993; accepted 5 January 1994)

The proteolytic formation of the cocoa-specific aroma precursors was studied *in vitro* using the vicilin-class globulin from cocoa seeds as protein substrate and different proteases. Cocoa-specific aroma precursors were obtained by degradation of the cocoa globulin with the aspartic endoprotease from cocoa seeds followed by post-treatment with carboxypeptidase A from porcine pancreas. Proteolysis products derived from the cocoa globulin by successive digestion with pepsin and carboxypeptidase A also revealed a typical, but less pronounced cocoa aroma upon roasting in the presence of reducing sugars. No cocoa-specific aroma precursors were, however, generated by degradation of the cocoa globulin with chymotrypsin and carboxypeptidase A. Therefore, the specific mixture of oligopeptides and hydrophobic free amino acids required for the formation of the typical cocoa aroma components is not only determined by the structure of the protein substrate but also dependent on the specificity of the endoprotease.

INTRODUCTION

The typical cocoa aroma is formed on roasting of fermented but not of unfermented cocoa seeds (Rohan, 1964). Mohr *et al.* (1971, 1976) have fractionated and partially characterised the essential precursors of the cocoa-specific aroma components present in fermented cocoa seeds. These authors have shown that a mixture of free amino acids and an oligopeptide fraction isolated from fermented cocoa seeds has to be roasted in the presence of reducing sugars to get cocoa-specific aroma (Mohr *et al.*, 1976). These aroma-related oligopeptides and free amino acids are formed during fermentation of the cocoa seeds by acid-induced proteolysis of seed proteins by endogenous proteases of the cocoa seeds (Passern, 1979; Biehl *et al.*, 1982, 1985; Biehl & Passern, 1982; Ziegleder & Biehl, 1988). Microbial degradation of sugars in the pulp results in the generation of acids (Quesnel, 1957, 1965)—predominantly acetic acid—which cause acidification and proteolytic processes in the nib Biehl *et al.*, 1985). Acid-induced proteolysis and formation of cocoa-specific aroma precursors have been shown to occur during incubation of cocoa seeds under laboratory conditions in the absence of microorganisms (Biehl *et al.*, 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). A correlation has been found between the pH-dependencies of aroma precursor formation (Biehl *et al.*, 1985) and of the liberation of free amino acids (Kirchhoff *et al.*, 1989*a,b*).

Cocoa-specific aroma precursors have been produced *in vitro* by autolysis at pH 5·2 of acetone dry powder prepared from unfermented cocoa seeds (Voigt *et al.*, 1993*a*; 1994*a*). Hydrophilic oligopeptides and hydrophobic free amino acids—predominantly Leu, Phe, Ala, Val and Tyr—are formed under these conditions. At pH 3·5, the pH-optimum of the predominant (aspartic) endoprotease present in ungerminated cocoa seeds (Biehl *et al.*, 1982, 1993*a,b*), no cocoa-specific aroma precursors are formed (Voigt *et al.*, 1993*a*; 1994*a*). Only trifling amounts of free amino acids and hydrophilic peptides, but large amounts of hydrophobic oligopeptides are generated under these conditions. Cocoa-specific aroma precursors are obtained when these hydrophobic peptides are transformed to mixtures of hydrophilic oligopeptides and hydrophobic-free amino acids by treatment with carboxypeptidase A from porcine pancreas Voigt *et al.*, 1993*a*; 1994*a*). The latter findings indicate that the aspartic endoprotease and the carboxypeptidase present in ungerminated cocoa seeds are involved in the proteolytic formation of the cocoa-specific aroma precursors. This conclusion has been

further corroborated by degradation of purified seed proteins with proteases partially purified from ungerminated cocoa seeds (Voigt *et al.*, 1994*b*). Cocoa-specific aroma precursors have only been obtained when the globular storage protein of the cocoa seeds has been successively degraded with the aspartic endoprotease and the carboxypeptidase isolated from cocoa seeds.

The successful production of the cocoa-specific aroma precursors by *in vitro* proteolysis of seed proteins has enabled us to investigate the significance of protein substrate structure and protease specificity in this process. As recently shown, no cocoa-specific aroma precursors are generated when the predominant cocoa seed albumin or globular storage proteins from coconuts, hazelnuts and sunflower seeds, respectively, are degraded by the aspartic endoprotease and the carboxypeptidase from ungerminated cocoa seeds (Voigt *et al.*, 1994*b,c*). These findings indicate that the essential mixture of hydrophobic free amino acids and hydrophilic oligopeptides required for the generation of the typical cocoa aroma components is determined by the particular chemical structure of the cocoa globulin. However, the specificity of the endoprotease is also important in this process 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 10g 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 acetone dry powder

Acetone dry powder (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 13 000 × *g*). After the final extraction step, efficiency of polyphenol extraction was checked by heating an aliquot of the AcDP with 5 M HCl (a 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 AcDP was stored at −20°C.

Fractionation of cocoa seed proteins and isolation of the globular storage protein

Seed proteins were extracted from polyphenol-free AcDP as recently described (Voigt *et al.*, 1993*b*). AcDP (50 g) was first extracted with 5 litre 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 20 000 × g and 4°C in the Sorvall GSA rotor. The extraction was repeated and the supernatants combined.

The vicilin-class globulin of the cocoa seeds (McHenry & Fritz, 1992; Spencer & Hodge, 1992; Voigt *et al.*, 1993*b*) was isolated from the crude globulin fraction as described by Pettipher (1990). The combined high-salt extracts were dialysed against distilled water and subsequently against 20 mM sodium acetate (pH 5·0). The precipitated globulins were collected by centrifugation at 20 000 *g* × for 30 min, washed with distilled water and stored at −20°C until use.

Preparation of the aspartic endoprotease from ungerminated cocoa seeds

The low-salt extract (equivalent to the crude albumin fraction), prepared from ungerminated cocoa seeds, 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) as recently described (Voigt *et al.*, 1994*b*). The low-salt extract stored at —70°C was thawed and 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 (Voigt *et al.*, 1994*a,b*). The aspartic endoprotease was eluted between 330 and 390 mM NaCl. Peak fractions of the aspartic endoprotease activity were combined, concentrated by treatment with Aquacide II (Calbiochem-Behring, Marburg, Germany), dialysed against 10 mM sodium phosphate (pH 7·5) and stored at −20°C.

Determination of aspartic endoprotease activity

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 stopped by addition of 0·2 ml of trichloroacetic acid (25%, w/v). The precipitated protein was removed by centrifugation at 10 000 × g for 15 min. Subsequently, proteolysis was measured colorimetrically by the trinitrobenzene–sulphonic acid method as described by Shutov *et al.* (1982). Each sample was analysed for protease activity in the presence or 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.

Determination of protein

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

Electrophoresis

Sodium dodecyl sulphate—polyacrylamide gel electrophoresis, (SDS-PAGE) was performed on gel slabs (140 mm × 140 mm × 1·5 mm) according to Laemmli (1970). The protein fractions to be analysed 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-mercaptoethanol (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 brilliant blue R250.

Digestion of purified cocoa globulin with different proteases

Digestion with aspartic endoprotease from cocoa seeds
Purified cocoa globulin (5 g) was suspended in 2 litres of distilled water. After addition of 100 mg of partially purified aspartic endoprotease (0·5 units/mg), the solution was adjusted to 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 20 000 × *g* for 30 min. The supernatants were collected and the methanol removed under reduced pressure at 40°C by means of a rotary evaporator.

Digestion with pepsin
A suspension of 5 g of cocoa globulin in 2 liters of distilled water was adjusted to pH 3·5 after addition of 50 mg of pepsin, the suspension was incubated at 37°C in a shaking water bath. After 3 h, another portion of pepsin (50 mg) was added and the incubation continued for 16 h. After addition of methanol to a final proportion of 70% (v/v), the suspension was stirred at room temperature for 1 h and then centrifuged at 20 000 × *g* for 30 min to remove the precipitates. The supernatant was concentrated at 40°C under reduced pressure by use of a rotary evaporator.

Digestion with chymotrypsin
Diluted NaOH was added to a suspension of 5 g of cocoa globulin in 2 litres of distilled water to achieve a pH value of 7·5. After addition of 50 mg of chymotrypsin, the suspension was incubated in a shaking water bath at 37°C. After 3 h, another portion of chymotrypsin (50 mg) was added and the incubation continued for 16 h. After addition of methanol to a final proportion of 70% (v/v), the suspension was stirred at room temperature for 1 h and subsequently centrifuged at 20 000 × *g* for 30 min. The supernatants were combined and the methanol removed at 40°C under reduced pressure by use of a rotary evaporator.

Post-treatment of oligopeptide mixtures with carboxypeptidase A
Oligopeptide mixtures obtained by proteolysis of purified cocoa globulin with either aspartic endoprotease from cocoa seeds, pepsin or chymotrypsin as described above were adjusted to pH 6·5. After addition of 300 units of commercial carboxypeptidase A from porcine pancreas, the solutions were incubated at 37°C in a shaking water bath. After 3 h, another 300 units of carboxypeptidase A 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 12 test persons. The sensory evaluation of

the aromas obtained was restricted to a qualitative identification of aroma notes. The proportion of test persons recognising 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*a*) using the HPLC system Gold (Beckman Instruments, San Ramon, CA, USA) equipped with an Ultrasphere ODS 5 μm column (4·6 mm × 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 × 4·6 mm) column and a Shandon Hypersil ODS 10 (20 mm × 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).

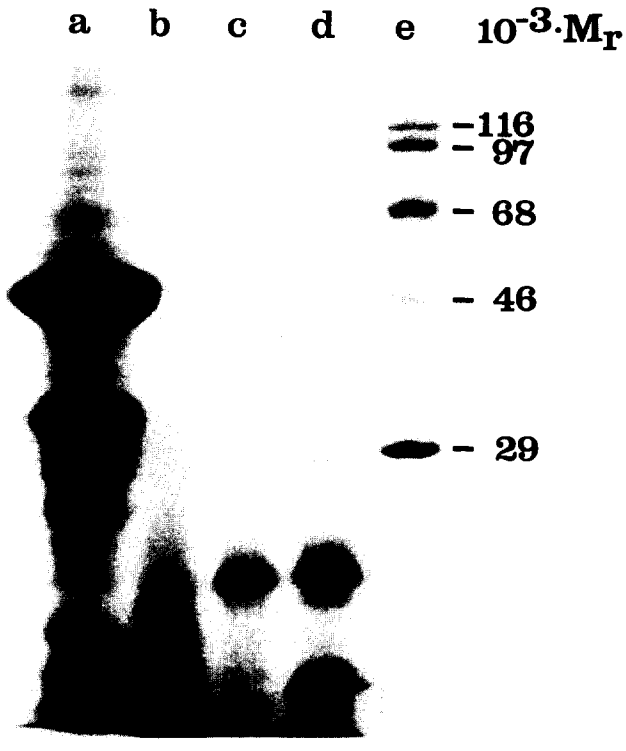

Fig. 1. SDS-PAGE analysis of the products formed during incubation of the globulin from cocoa seeds in the absence and presence of aspartic endoprotease from cocoa seeds, pepsin and chymotrypsin, respectively. The purified cocoa globulin was incubated at pH 3·5 and 50°C in the absence (lane a) or presence of aspartic endoprotease (lane b), at pH 3·5 and 37°C in the presence of pepsin (lane c) or at pH 7·5 and 37°C in the presence of chymotrypsin (lane d) as described in the 'Materials and Methods'. 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 brilliant blue. The molecular weights (M_r) of protein standards (lane e: β-glucosidase = 116 000; phosphorylase b = 97 000; bovine serum albumin = 68 000; ovalbumin = 46 000; carbonic anhydrase = 29 000) are indicated by the numbers on the right.

RESULTS

Cocoa-specific aroma precursors can be produced *in vitro* by proteolysis of the vicilin-class globulin from cocoa seeds with the aspartic endoprotease and the carboxypeptidase isolated from ungerminated cocoa seeds (Voigt *et al.*, 1994*a,b*). The carboxypeptidase from cocoa seeds can be substituted by carboxypeptidase A from porcine pancreas in this process (Voigt *et al.*, 1993*a*, 1994*a*).

The aspartic endoprotease from cocoa seeds cleaves protein substrates at hydrophobic amino acid residues to produce oligopeptides with hydrophobic amino acid residues at their carboxyterminal ends (Voigt *et al.*, 1994*a*). Therefore, we have investigated whether or not cocoa-specific aroma precursors can be obtained by proteolysis of the cocoa globulin with proteases from different origin, but similar specificity as compared to the cocoa aspartic endoprotease. In a first series of experiments, we have used pepsin, an aspartic endoprotease with a broad specificity for hydrophobic amino acid residues (Fruton, 1970; Ryle, 1970; Sampath-Kumar & Fruton, 1974), and chymotrypsin, a serine endoprotease whose specificity is restricted to aromatic amino acid residues (Wilcox, 1970; Blow, 1971).

The vicilin-class globulin isolated from the cocoa seeds (Voigt *et al.*, 1993*b*) was found to be efficiently degraded by aspartic endoprotease from cocoa seeds, pepsin and chymotrypsin, respectively (Fig. 1). Comparative reversed-phase HPLC analyses of the obtained proteolysis products revealed that considerably different patterns of oligopeptides had been formed during degradation of the cocoa globulin with aspartic endoprotease from cocoa seeds (Fig. 2(A)), pepsin (Fig. 2(B)) and chymotrypsin (Fig. 2(C)), respectively. The bulk of the chymotryptic peptides (Fig. 2(C)) eluted at lower acetonitrile concentrations than most of the oligopeptides generated by the action of aspartic endoprotease from cocoa seeds (Fig. 2(A)) or pepsin (Fig. 2(B)). However, differences were also observed between the oligopeptide patterns obtained by degradation of the cocoa globulin with aspartic endoprotease from cocoa seeds (Fig. 2(A)) and pepsin (Fig. 2(B)), respectively.

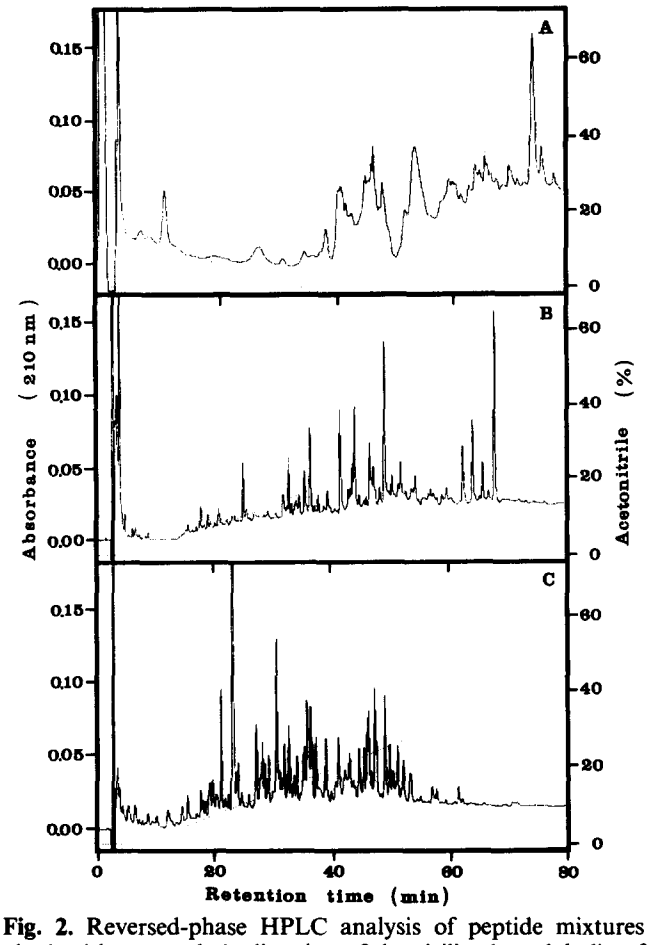

Fig. 2. Reversed-phase HPLC analysis of peptide mixtures obtained by proteolytic digestion of the vicilin-class globulin of cocoa seeds with different endoproteases. The cocoa globulin was digested at pH 3·5 with aspartic endoprotease of cocoa seeds (A) and pepsin (B), respectively, or at pH 7·5 with chymotrypsin (C) and the resulting peptide mixtures analysed by reversed-phase HPLC as described in the 'Materials and Methods'.

When these oligopeptide mixtures were treated with carboxypeptidase A from porcine pancreas, hydrophobic amino acids were preferentially released from all the peptide mixtures. However, the relative amounts of the various hydrophobic amino acids was found to be different (Table 1). In the case of the chymotryptic peptides, the aromatic amino acids were preferentially liberated during incubation with carboxypeptidase A. From the oligopeptide mixtures generated by pepsin and aspartic endoprotease from cocoa seeds, respectively, considerably higher amounts of Ala and especially Leu were released (Table 1). Reversed-phase HPLC of the carboxypeptidase-treated oligopeptides revealed considerably less complex patterns (Fig. 3) as compared to the oligopeptide patterns observed prior to carboxypeptidase treatment (Fig. 2). The carboxypeptidase-modified peptides eluted in a few peaks at relatively low acetonitrile concentration (Figs 3(A)—(C)). Rather similar elution profiles were obtained for the carboxypeptidase-modified oligopeptide mixtures generated by the aspartic endoprotease from cocoa seeds Fig. 3(A)), pepsin (Fig. 3(B)) and chymotrypsin (Fig. 3(C)), respectively.

The proteolysis products derived from the cocoa

Table 1. Amino acids released by carboxypeptidase A from the oligopeptide mixtures generated *in vitro* by degradation of the vicilin-class globulin from cocoa seeds with chymotrypsin, pepsin and aspartic endoprotease from cocoa seeds, respectively[a]

Amino acid	Cocoa aspartic endoprotease	Pepsin	Chymotrypsin
Asp	34·7	19·0	23·4
Glu	41·2	37·3	38·1
Asn	54·0	38·9	35·8
Ser	32·8	28·1	39·6
Gln + His	63·5	34·3	47·2
Gly	16·3	13·1	13·9
Thr	39·4	17·9	18·7
Arg	15·3	16·7	12·8
Ala	105·7	38·9	95·5
Tyr	61·9	50·7	79·4
Trp	9·1	7·1	3·3
Met	35·2	19·6	18·5
Val	98·4	50·6	65·5
Phe	168·1	112·2	178·6
Ile	55·3	38·3	47·3
Leu	159·35	132·8	80·5
Lys	1·2	3·3	1·8

[a] The globulin prepared from cocoa seeds was digested with chymotrypsin at pH 7·5 and 37°C, with pepsin at pH 3·5 and 37°C and with aspartic endoprotease from cocoa seeds at pH 3·5 and 50°C, respectively. The obtained oligopeptide mixtures were adjusted to pH 6·5 and post-treated at 37°C with carboxypeptidase A from porcine pancreas as described in the 'Materials and Methods'. Free amino acids present in the proteolysis products were analysed by reversed-phase HPLC of the OPA derivatives (Kirchhoff *et al.*, 1989a). Without carboxypeptidase treatment, very low levels of free amino acids (between 0 and 2 nmol/mg dry weight) were found. Values are given in nmol/mg dry weight.

Table 2. Cocoa-specific aroma potentials of proteolysis products generated *in vitro* from the vicilin-like globulin from cocoa seeds by digestion with aspartic endoprotease from cocoa seeds, chymotrypsin and pepsin, respectively, and subsequent post-treatment of the resulting oligopeptide mixtures with carboxypeptidase A from porcine pancreas

Endoprotease[a]	Cocoa-specific aroma potential[b,c]	
Aspartic endoprotease from cocoa seeds	6/12	(50%)
Pepsin	3/12	(25%)
Chymotrypsin	0/12	(0%)

[a] The vicilin-class globulin prepared from cocoa seeds was digested with aspartic endoprotease from cocoa seeds, pepsin and chymotrypsin, respectively, and the generated oligopeptide mixtures post-treated with carboxypeptidase A from porcine pancreas as described in the 'Materials and Methods'.

[b] The freeze-dried protoeolysis products were formulated and roasted in the presence of reducing sugars as described in the 'Materials and Methods'. Sensory evaluation 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. Percentage of test persons who have recognised cocoa and/or chocolate aroma are shown in parentheses.

[c] Cocoa or chocolate aroma was never obtained by roasting of oligopeptide mixtures which were not post-treated with carboxypeptidase A.

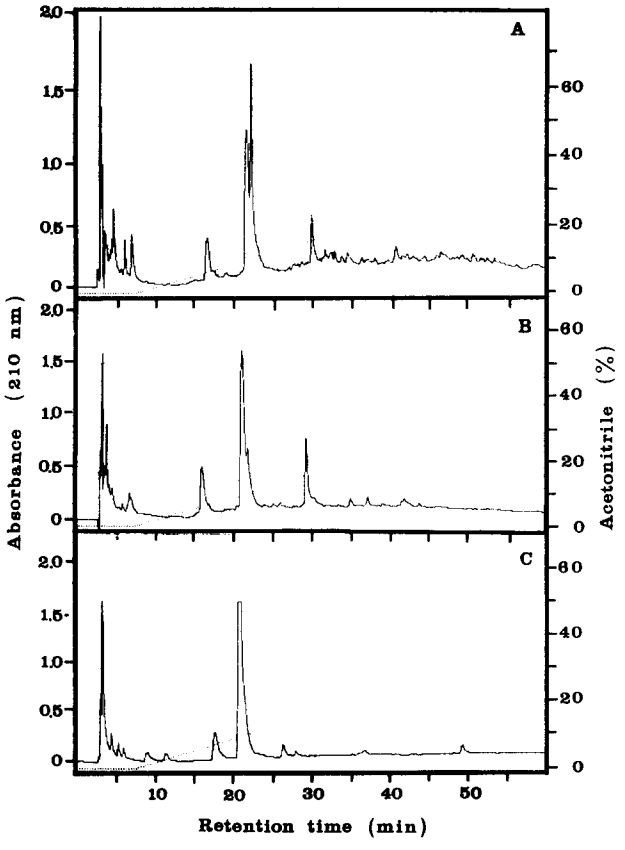

Fig. 3. Reversed-phase HPLC analysis of peptide mixtures obtained by proteolytic digestion of the cocoa globulin with different endoproteases and subsequent post-treatment with carboxypeptidase A from porcine pancreas. The cocoa globulin was digested with aspartic endoprotease from cocoa seeds, pepsin and chymotrypsin, respectively, under conditions optimum for the used enzymes (see the 'Materials and Methods'). The obtained oligopeptide mixtures were post-treated with carboxypeptidase A from porcine pancreas and the resulting peptide mixtures analysed by reversed-phase HPLC as described in the 'Materials and Methods'. (A) Cocoa globulin digested with aspartic endoprotease from cocoa seeds and post-treatment of the generated oligopeptides with carboxypeptidase A from porcine pancreas; (B) cocoa globulin digested with pepsin and post-treatment of the resulting oligopeptides with carboxypeptidase A; (C) cocoa globulin digested with chymotrypsin and post-treatment of the generated oligopeptides with carboxypeptidase A.

globulin by degradation with aspartic endoprotease from cocoa seeds, pepsin and chymotrypsin, respectively, and subsequent post-treatment with carboxypeptidase A from porcine pancreas, were formulated and roasted in the presence of reducing sugars and deodorized cocoa butter. The obtained roasting aromas were sensorially evaluated by a panel of 12 test persons. As shown in Table 2, 50% of the test persons recognised cocoa and chocolate aroma in the case of the proteolysis products derived from the cocoa globulin by degradation with the aspartic endoprotease from cocoa seeds and carboxypeptidase from porcine pancreas. No cocoa or chocolate aroma was obtained by roasting of the chymotryptic peptides post-treated with carboxypeptidase A (Table 2). When proteolysis products derived from the cocoa globulin by degradation with pepsin and post-treatment with carboxypeptidase A were roasted in the presence of reducing sugars, three out of 12 test persons recognized cocoa and chocolate aroma. These findings indicate that the specificity of the endoprotease is important for the formation of the cocoa-specific aroma precursors.

DISCUSSION

During the last 30 years, it has been shown that the cocoa-specific aroma precursors are formed by proteolytic processes which occur during fermentation of the cocoa seeds (Rohan, 1964; Mohr *et al.*, 1971, 1976) and which are not directly dependent on the presence of microorganisms, but are induced by acidification of the seeds (Biehl *et al.*, 1985). Furthermore, it has been found that the generation of these typical aroma precursors is strongly dependent on the degree and the time course of the acidification of the cocoa seeds (Biehl *et al.*, 1985) and correlates with the liberation of specific hydrophobic amino acids in the cocoa seeds (Kirchhoff *et al.*, 1989*a,b*). These observations have led to the development of a procedure (post-harvest storage of cocoa pods) that has improved raw-cocoa quality in Malaysia (Biehl *et al.*, 1989; Meyer *et al.*, 1989).

Recently, the authors have successfully tried to produce the cocoa-specific aroma precursors by acid-induced proteolysis of cocoa seed proteins *in vitro* (Voigt *et al.*, 1993*a*; 1994*a*). Using this experimental approach, we have been able to show that the cocoa-specific aroma precursors are derived from the vicilin-class globulin present in the cocoa seeds (Voigt *et al.*, 1993*b*, 1994*b,c*) and that this cocoa globulin cannot be substituted by the globular storage proteins present in coconuts, hazelnuts or sunflower seeds, (Voigt *et al.*, 1994*c*). Two proteases are involved in this process: the aspartic endoprotease and the carboxypeptidase present in ungerminated cocoa seeds (Biehl *et al.*, 1993*a*; Voigt *et al.* 1994*a*). It has been shown that the carboxypeptidase from cocoa seeds can be substituted by carboxy peptidase A from porcine pancreas (Voigt *et al.*, 1993*a,b*; this paper. Both carboxypeptidases have a similar substrate specificity. They preferentially cleave off hydrophobic amino acid residues and are not able to release carboxyterminal Arg, Lys and Pro residues (Neurath, 1960; Ambler, 1972; Biehl *et al.*, 1993*b*; Bytof, 1993).

The aspartic endoprotease cleaves protein substrates to produce oligopeptides with hydrophobic amino acid residues at their carboxyterminal ends (Voigt *et al.*, 1994*a*, 1994*b,c*). Therefore, the authors have investigated whether or not cocoa-specific aroma precursors are obtained when the vicilin-class globulin of the cocoa seeds is degraded by endoproteases from other origins but similar substrate specificity, and post-treatment of the resulting oligopeptides with carboxypeptidase A from porcine pancreas. Pepsin, but not chymotrypsin has been found to be a suitable endoprotease for this purpose. However, the mixture of oligopeptides and hydrophobic free amino acids derived from the cocoa globulin by the action of pepsin and

carboxypeptidase A revealed a considerably less pronounced cocoa aroma on roasting in the presence of reducing sugars than the proteolysis products generated by degradation of the cocoa globulin with the aspartic endoprotease from cocoa seeds and post-treatment with carboxypeptidase A. These findings indicate that both aspartic endoproteases (Fruton, 1970; Ryle, 1970; Samath-Kumar & Fruton, 1974; Biehl *et al.*, 1993*a*) have a similar but not the same specificity. This conclusion has been corroborated by comparative reversed-phase HPLC analyses of the oligopeptide patterns generated by both endoproteases (Fig. 2). The results clearly show that the particular specificity of the aspartic endoprotease of the cocoa seeds is important for the formation of the cocoa-specific aroma precursors.

ACKNOWLEDGMENTS

The authors are grateful to Dr S. Kamaruddin (Cocoa and Coconut Research Division, Hilir Perak, Malaysia, of the Malaysian Agricultural Research and Development Institute) for providing fresh cocoa pods at our disposal and to Dr A. Hugi and Mr G. Gaim Marsoner (Jacobs-Suchard, Research et Development, Neuchatel, Switzerland) for their help in the establishment of the formulation and roasting procedures and the sensory evaluation of roasting aromas. This work has been supported by a grant (AIF 8427) from the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen e.V./Forschungskreis der Ernährungsindustrie (FEI).

REFERENCES

Ambler, R. P. (1972). Enzymatic hydrolysis with carboxypeptidases. *Meth. Enzymol.*, **25**, 143–54.

Bennett, H. P. J., Browne, C. A. & Solomon, S. (1980). The use of perfluorinated carboxylic acids in the reversed phase HPLC of peptides. *J. Liquid Chromatogr.*, **3**, 1353–65.

Biehl, B. & Passern, D. (1982). Proteolysis during fermentation-like incubation of cocoa seeds. *J. Sci. Food Agric.*, **33**, 1280–90.

Biehl, B., Wewetzer, C. & Passern, D. (1982). Vacuolar (storage) proteins of cocoa seeds and their degradation during germination and fermentation. *J. Sci. Food Agric.*, **33**, 1291–304.

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**, 583–98.

Biehl, B., Meyer, B., Crone, G. & Pollmann, L. (1989). Chemical and physical changes in the pulp during ripening and post harvest storage of cocoa pods. *J. Sci. Food Agric.*, **48**, 189–208 .

Biehl, B., Heinrichs, H., Ziegeler-Berghausen, H., Hammoor, M. & Senyuk, V. (1993*a*). The proteases of ungerminated cocoa seeds and their role in the fermentation process. *Angew. Bot.*, **67**, 59–65.

Biehl, B., Voigt, J., Voigt, G., Heinrichs, H., Senyuk, V. & Bytof, G. (1993*b*). pH-Dependent enzymatic formation of oligopeptides and amino acids, the aroma precursors in raw cocoa beans. In *Proceedings of the 11th International Cocoa Research Conference*, 18–24 July 1993, Yamoussoukro, Ivory Coast (in press).

Blow, D. M. (1971). The structure of chymotrypsin. In *The Enzymes* (Vol. 3), ed. P. D. Bpyer, Academic Press, New York, NY, USA, pp. 185–212.

Bytof, G. (1993). Carboxypeptidase im Samen von *Theobroma cacao* L. Diplomarbeit, Technische Universität Braunschweig, Germany.

Fruton, J. S., (1970). Specificity and mechanism of pepsin action. *Adv. Enzymol.*, **33**, 401–43.

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

Kirchhoff, P.-M., Biehl, B., Ziegeler-Berghausen, H., Hammoor, M. & Lieberei, R. (1989*b*). Kinetics of the formation of free amino acids in cocoa seeds during fermentation. *Food Chem.*, **34**, 161–79.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–5.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurements with the folin phenol reagent. *J. Biol. Chem.*, **193**, 257–65.

Mahoney, W. C. & Hermodson, M. A. (1980). Separation of large denaturated peptides by reversed phase high performance liquid chromatography. Trifluoroacetic acid as a peptide solvent. *J. Biol. Chem.*, **255**, 11199–203.

McHenry, L. & Fritz, P. J. (1992). Comparison of the structure and nucleotide sequence of vicilin genes of cocoa and cotton raise questions about vicilin evolution. *Plant Mol. Biol.*, **18**, 1173–6.

Meyer, B., Biehl, B., Said, M. B. & Samarakoddy, R. J. (1989). Post harvest pod storage: A method for pulp preconditioning to impair strong nib acidification during cocoa fermentation in Malaysia. *J. Sci. Food Agric.*, **48**, 285–304.

Mohr, W., Röhrle, M. & Severin, Th. (1971). Über die Bildung des Kakaoaromas aus seinen Vorstufen. *Fette, Seifen, Anstrichmittel*, **73**, 515–21.

Mohr, W., Landschreiber, E. & Severin, Th. (1976). Zur Spezifität des Kakaoaromas. Fette, Seifen, Anstrichmittel, **78**, 88–95.

Neurath, H. (1960). Carboxypeptidase A. In *The Enzymes* (Vol. 4), eds P. D. Boyer, H. A. Lardy & K. Myrbäck. Academic Press, New York, NY, USA, p. 11.

Passern, D. (1979). Zur Aktivität und zellulären Kontrolle saurer Proteasen während Samenentwicklung, Keimung und Fermentation von Kakaosamen (*Theobroma cacao* L.). Dissertation, Technische Universität Braunschweig, Germany.

Pettipher, G. L. (1990). The extraction and partial purification of cocoa storage proteins. *Café Cocoa Thé*, **34**, 23–26.

Quesnel, V. C. (1957). *Curing Cocoa in the Laboratory.* Rep. Cocoa Conf., Cocoa, Chocolate and Confectionery Alliance, London, UK, pp. 150–6.

Quesnel, V. C. (1965). Agents inducing the death of cocoa seeds during fermentation. *J. Sci. Food Agric.*, **16**, 441–7.

Rohan, T. A. (1964). The precursors of chocolate aroma: a comparative study of fermented and unfermented cocoa beans. *J. Food Sci.*, **29**, 456–9.

Ryle, A. P. (1970). The porcine pepsins and pepsinogens. *Methods Enzymol.*, **19**, 316–36.

Sampath-Kumar, P. S & Fruton, J. S (1974). Extended active sites of acid proteinases. *Proc. Nat. Acad. Sci. USA*, **71**, 1070–2.

Shutov, A. D., Lanh, D. N. & Vaintraub, I. A. (1982). Purification and partial characterization of protease B from germinating vetch seeds. *Biokhimya*, **47**, 814–21.

Spencer, M. E. & Hodge, R. (1992). Cloning and sequencing of a cDNA encoding the major storage proteins of *Theobroma cacao*. Identification of the proteins as members of the vicilin class of storage proteins. *Planta*, **186**, 567–76.

Voigt, J. (1985). Extraction by lithium chloride of hydroxyproline-rich glycoproteins from intact cells of *Chlamydomonas reinhardtii*. *Planta*, **164**, 379–89.

Voigt, J., Biehl, B. & Heinrichs, H. (1993*a*). Proteolytic for-

mation of cocoa flavour precursors. In *Progress in Flavour Precursors Studies*, ed. P. Schreier. Allured Publishing Corp., Wheaton, USA, pp. 213–6.

Voigt, J., Biehl, B. & Kamaruddin, S. (1993*b*). The major seed proteins of *Theobroma cacao* L. *Food Chem.*, **47**, 145–51.

Voigt, J., Biehl, B., Heinrichs, H., Kamaruddin, S., Gaim Marsoner, G. & Hugi, A. (1994*a*). *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**, 173–80.

Voigt, J., Heinrichs, H., Voigt, G. & Biehl, B. (1994*b*). Cocoa-specific aroma precursors are generated by proteolytic digestion of the vicilin-like globulin of the cocoa seeds. *Food Chem.*, **50**, 177–84.

Voigt, J., Wrann, D., Heinrichs, H. & Biehl, B. (1994*c*). The proteolytic formation of essential cocoa-specific aroma precursors depends on particular chemical structures of the cocoa globulin lacking in globular storage proteins of other plants. *Food Chem.*, (in press).

Wilcox, P. E. (1970). Chymotrypsinogens—chymotrypsins. *Methods Enzymol.*, **19**, 64–108.

Ziegleder, G. & Biehl, B. (1988). Analysis of cocoa flavour components and flavour precursors. *Methods in Plant Analysis* (Vol. 8, new series) eds H. Linkens & J. F. Jackson. Springer-Verlag, Berlin, Germany, pp. 321–93.