ELSEVIER

*Food Chemistry* 51 (1994) 197–205

0308-8146/94/$7.00

# The proteolytic formation of essential cocoa-specific aroma precursors 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

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

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

(Received 20 October 1993; revised version received and accepted 4 January 1994)

Cocoa-specific aroma precursors were generated *in vitro*, when the vicilin-class globulin of cocoa seeds was successively degraded by the aspartic endoprotease and the carboxypeptidase isolated from ungerminated cocoa seeds. To study the significance of the chemical structure of the protein substrate, globular storage proteins were isolated from different crops and subjected to proteolysis by the aspartic endoprotease and the carboxypeptidase of ungerminated cocoa seeds. The obtained proteolysis products were comparatively analysed for their patterns of oligopeptides and free amino acids and were roasted in the presence of reducing sugars and deodorised cocoa butter. Sensory evaluation of the roasting aromas revealed that neither the proteolysis products of the legumin-class globulins from hazelnuts or sunflower seeds, nor those derived from the vicilin-class globulin of coconuts contained the typical pattern of aroma precursors formed by degradation of the vicilin-class globulin of cocoa seeds with the same proteases. Considerable differences were observed between the patterns of free amino acids and oligopeptides generated by proteolysis of the cocoa vicilin and of the globular storage proteins from the other crops. These findings indicate that the formation of the cocoa-specific aroma precursors is determined by the particular chemical structure of the vicilin-class globulin present in the cocoa seeds.

## INTRODUCTION

In the past 24 years evidence shows that essential precursors of the cocoa-specific aroma components are generated during fermentation of cocoa seeds by an acid-induced proteolysis of seed proteins by endogenous proteases (Rohan, 1964; Mohr *et al.*, 1971, 1976; Biehl *et al.*, 1982, 1985; Ziegleder & Biehl, 1988). Cocoa aroma is developed on roasting of the fermented but not of unfermented cocoa seeds (Rohan, 1964). Investigating the cocoa-specific aroma precursors present in fermented cocoa seeds, Mohr *et al.* (1976) have isolated an oligopeptide fraction which revealed typical cocoa aroma when roasted in the presence of free amino acids and reducing sugars. Both the proteolysis of seed proteins and the formation of essential aroma precursors are strongly dependent on the degree and the time course of nib acidification during the fermentation process (Biehl & Passern, 1982; Biehl *et al.* 1982, 1985), which is caused by microbial degradation of sugars in the pulp (Quesnel, 1957, 1965). 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 cocoa-specific aroma precursors (Biehl *et al.*, 1985). Strong acidification of the seeds results in raw cocoas with low aroma potentials (Biehl *et al.*, 1985). These findings 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).

Recently, the authors were able to produce the cocoa-specific aroma precursors by *in vitro* proteolysis of seed proteins with proteases from ungerminated cocoa seeds (Voigt *et al.*, 1993*a*, 1994*a*,*b*). Using this experimental approach, we have shown that the aspartic endoprotease and the carboxypeptidase present in ungerminated cocoa seeds (Biehl *et al.*, 1991, 1993) are involved in the generation of the typical aroma precursors (Voigt *et al.*, 1993*a*, 1994*a*,*b*). As reported by Pettipher and co-workers (Macdonald *et al.*, 1991), no cocoa-specific aroma precursors have been obtained

when cocoa seed proteins have been solely digested with endoproteases of different specificity. Cocoa seeds contain two major proteins (Voigt *et al.*, 1993*b*): a 19 kDa albumin which is related to the soya bean trypsin inhibitor (Kunitz) family of protease inhibitors (Spencer & Hodge, 1991; Tai *et al.*, 1991) and a vicilin-class globulin (Spencer & Hodge, 1992; McHenry & Fritz, 1992; Voigt *et al.* 1993*b*). The vicilin-class globulin, but not the albumin, is a protein substrate suitable for the proteolytic formation of the cocoa-specific aroma precursors (Voigt *et al.*, 1994*b*). Therefore, the authors have investigated whether or not cocoa-specific aroma precursors can be derived from globular storage proteins of other crops by successive degradation with the aspartic endoprotease and the carboxypeptidase from ungerminated cocoa seeds.

## 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. Coconuts, hazelnuts and sunflower seeds were commercially obtained and genetically undefined.

### Extraction of fat

The freeze-dried seeds were crushed and portions of 10 g each were extracted repeatedly in a Soxhlet apparatus with 500 ml of petroleum ether (b.p. 40–70°C). After solvent evaporation, the material was powdered and extracted again for 8 h in the same manner. In the case of the cocoa seeds, purine alkaloids were partially extracted with chloroform for 8 h in a Soxhlet apparatus.

### Preparation of acetone dry powder (AcDP)

Acetone dry powders (AcDP) were prepared essentially as recently described for cocoa seed powder (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 x 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.

### Isolation of globular storage proteins

Seed proteins were extracted from polyphenol-free AcDP as recently described (Voigt *et al.* 1993*b*): 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 crude 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 high-salt extract was 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 aspartic endoprotease and carboxypeptidase from ungerminated cocoa seeds

The low-salt extract (= crude albumin fraction) prepared from ungerminated cocoa seeds was fractionated by anion-exchange chromatography using a Whatman DE-52 column (50 × 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 carboxypeptidase activity was eluted at the beginning of the salt gradient (100 mM NaCl) and, the aspartic endoprotease between 330 and 390 mM NaCl. Peak fractions of the carboxypeptidase and aspartic endoprotease activities, respectively, were combined, concentrated by treatment with Aquacide II (Calbio-chem-Behring, Marburg, Germany), dialysed 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 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 trinitrobenzenesulphonic 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/min 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 trinitrobenzenesulphonic acid method as described by Shutov *et al.* (1982). One unit of carboxpeptidase is the activity which releases 1 μmol leucine/min at pH 5·8 and 45°C.

**Determination of protein**

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

**Sucrose density gradients**

Sedimentation equilibrium analyses of globulins isolated from coconuts and hazelnuts, respectively, were accomplished on 10–30% sucrose gradients (in 0·5 M NaCl, 1 mM DTE, −20 mM Tris–HCl, pH 8·0) spun in a Beckman SW27 rotor at 64 000 × *g* for 24 h at 15°C. Sunflower globulin (12S), pea vicilin (7S) and bovine liver catalase (2·2 S) were used as calibration standards. Sunflower globulin and pea vicilin were prepared from the corresponding seeds as described in the literature (Derbyshire *et al.*, 1976). Bovine liver catalase was obtained from Sigma Chemie (Deisenhofen, Germany).

**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 and 20 mM Tris–HCl (pH 7·5) with or without 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, but lacking reducing agents like dithiothreitol or 2-mercaptoethanol, the samples were subjected to SDS-PAGE analysis. Gels were stained for protein with Coomassie brilliant blue R250.

**Proteolytic digestion of globular storage proteins with aspartic endoprotease and carboxypeptidase from cocoa seeds**

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 pH 5·2 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, incubations were stopped and the reaction mixtures stored at 4°C. Aliquots of the reaction mixtures were subjected to SDS-PAGE analysis to estimate the efficiency of the proteolysis.

When proteolysis of the proteins by the aspartic endoprotease was completed, the reaction mixtures were 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 of carboxypeptidase were added. Incubations were stopped after 16 h by addition of methanol to a final concentration of 70 % (v/v). The suspensions were stirred at room temperature for 1 h and the precipitates subsequently removed by centrifugation 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. Finally, the aqueous solutions 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. As a control, aroma precursors isolated from fermented

and air-dried cocoa seeds as described by Mohr *et al.* (1971, 1976) were analysed by the same procedure. 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 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. Only four to five out of 10 test persons recognised cocoa or chocolate aroma when aroma precursors isolated from fermented and air-dried cocoa seeds (Mohr *et al.*, 1971, 1976) were roasted in the presence of reducing sugars and deodorised cocoa butter.

### 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).

## RESULTS

As recently reported, cocoa-specific aroma precursors can be produced *in vitro*, when cocoa seed proteins are successively degraded by the aspartic endoprotease from cocoa seeds and either the carboxypeptidase from ungerminated cocoa seeds or carboxypeptidase A from porcine pancreas (Voigt *et al.*, 1993*a*, 1994*a*). Using purified cocoa seed proteins as substrates, we have shown that the globulin, but not the albumin of the cocoa seeds is a suitable substrate for the generation of the typical aroma precursors (Voigt *et al.*, 1994*b*). Therefore, the authors have investigated whether the

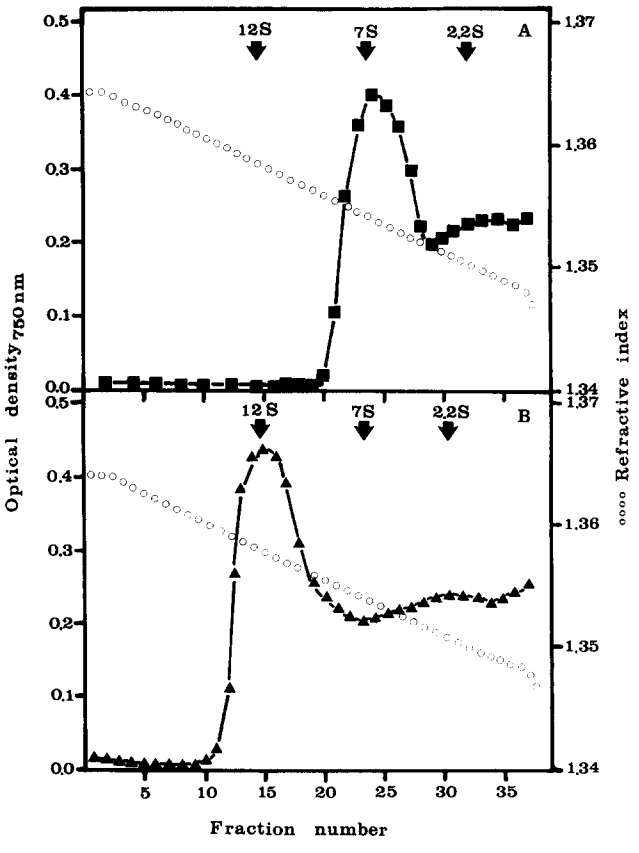


**Fig. 1.** Analytical sucrose gradient centrifugation of the globulin fractions of (A) coconuts and (B) hazelnuts. Globulin solutions isolated from acetone dry powders of coconuts and hazelnuts, respectively, were were layered onto 10–30% sucrose gradients and subjected to centrifugation at 68 000 × g for 20 h. The optical densities are based on Lowry absorbance (Lowry *et al.*, 1951) of 50 μl aliquots. The calibration standards are bovine liver catalase (2·2 S), pea vicilin (7 S) and sunflower globulin (12 S) sedimented as indicated by the arrows.

essential mixture of oligopeptides and free amino acids required for the formation of the cocoa-specific aroma components during the roasting process is determined by the particular chemical structure of the vicilin-class globulin of the cocoa seeds or whether this protein substrate can be substituted by globular storage proteins from other crops. For this purpose, globulins were isolated from coconuts, hazelnuts and sunflower seeds. Sunflower seeds have been reported to contain a predominant legumin-class (12S) globulin (Derbyshire *et al.*, 1976). The globulins present in coconuts and hazelnuts, however, have not yet been described in the literature. Therefore, we have first investigated the compositions of the globulin fractions prepared from coconuts and hazelnuts, respectively, by sucrose density gradient analysis (Fig. 1). Since polypeptide subunits of the legumin-class (11–12S) globulins, but not those of the vicilin-class (6–8S) globulins are cross-linked via disulphide bridges (Derbyshire *et al.*, 1976; Higgins, 1984; Müntz *et al.*, 1985; Shotwell & Larkins, 1988), we have also comparatively studied the polypeptide patterns of the globulin fractions in their non-reduced and reduced forms, respectively (Fig. 2).

Sucrose density gradient analysis of the globulin

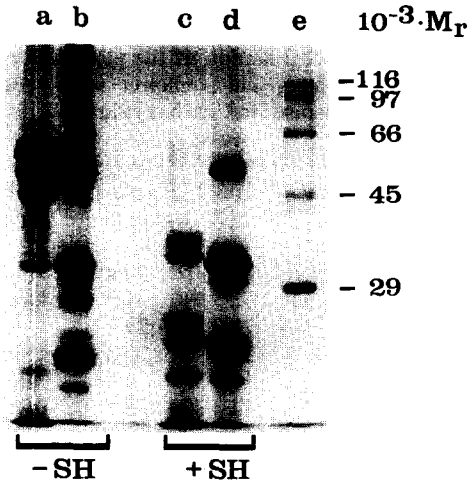


**Fig. 2.** Coomassie brilliant blue stained SDS-PAGE profiles of the globulin fractions from coconuts and hazelnuts run under non-reducing (−SH) and reducing conditions (+SH). Globulins were prepared from coconuts and hazelnuts in the absence of compounds which are able to reductively cleave disulphide bonds (as described in 'Materials and Methods'). The globulins were dissolved in SDS-containing sample buffer, incubated overnight in the absence (−SH) or presence of 200 mM 2-mercaptoethanol (+SH) and subjected to SDS-PAGE on gel slabs (140 × 140 × 0·15 mm) containing 15% (w/v) acrylamide according to Laemmli (1970). (Lane a) Hazelnut globulins, non-reduced form; (lane b) coconut globulin, non-reduced form; (lane c) hazelnut globulins, reduced form; (lane d) coconut globulin, reduced form; (lane e) protein molecular weight standard: β-galactosidase, 116 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa.

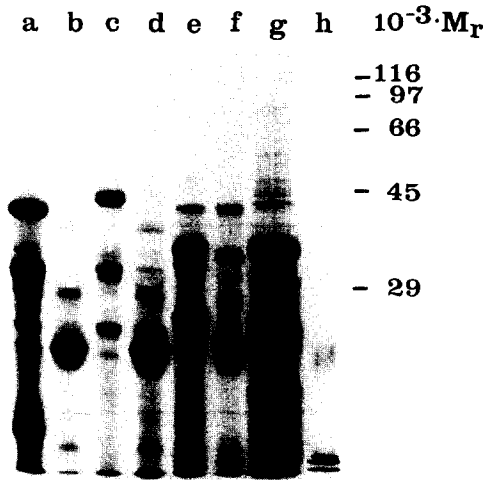


**Fig. 3.** SDS-PAGE analysis of the TCA-insoluble products formed during incubation of globular storage proteins from different plants in the presence and absence of aspartic endoprotease from cocoa seeds. Globulins prepared from acetone-dry powders of ungerminated cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, were incubated at pH 5·2 and 50°C in the absence or presence of aspartic endoprotease (20 μg per mg protein substrate) as described in 'Materials and Methods'. After 16 h, aliquots of the reaction mixtures corresponding to 70 μg initial protein were subjected to SDS-PAGE analysis according to Laemmli (1970). The gels were stained with Coomassie brilliant blue. The molecular weights of protein standards ($M_r$) are indicated by numbers on the right. (a, b) cocoa globulin incubated in the (a) absence or (b) presence of aspartic endoprotease; (c, d) coconut globulin incubated in the (c) absense or (d) presence of aspartic endoprotease (e, f) hazelnut globulin incubated in the (e) absence or (f) presence of aspartic endoprotease; (g, h) sunflower globulin incubated in the (g) absence or (h) presence of aspartic endoprotease.

fraction from coconuts revealed no 11–12S component, but a predominant peak in the 6–8 S region (Fig. 1(A)). The predominant component of the globulin fraction from hazelnuts, however, peaked in the 11–12 S region (Fig. 1(B)).

SDS-PAGE analysis of the non-reduced hazelnuts globulins revealed subunits with apparent molecular weights of 60 kDa, 54 kDa, 47 kDa, and 34 kDa (Fig. 2, lane a). These bands were not observed after reduction with 2-mercaptoethanol (Fig. 2, lane c). Instead, polypeptides with apparent molecular weights of 37 kDa, 33 kDa, 30 kDa, 24 kDa, 22 kDa and 18 kDa, respectively, were found in the reduced hazelnut globulin fraction (Fig. 2, lane c). These findings indicate that the globulin fraction from hazelnuts exclusively consists of legumin-class (11–12 S) globulins. The polypeptide pattern of the coconut globulin, however, was essentially not affected by treatment with 2-mercaptoethanol (Fig. 2, lanes b and d). Predominant polypeptides with apparent molecular weights of 54 kDa, 33 kDa, 20 kDa, and 19 kDa were found both in the non-reduced (Fig. 2, lane b) and in the reduced forms of the coconut globulin (Fig. 2, lane d). In accordance with the results of the sucrose density gradient analysis (Fig. 1(A)), it is concluded that the predominant component of the coconut globulin fraction is a vicilin-class (6-8 S) globulin.

The globulins isolated from cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, were treated for 16 h at pH 5·2 and 50°C with aspartic endoprotease from ungerminated cocoa seeds. Subsequently, aliquots were taken from the different incubation mixtures and subjected to SDS-PAGE analysis. As shown in Fig. 3, all the different globulins were found to be efficiently degraded by the cocoa aspartic endoprotease. When proteolysis of any of the globulins was incomplete, incubation of the corresponding sample was continued after a further addition of aspartic endoprotease.

Comparative HPLC analyses of the obtained peptide mixtures revealed different and complex patterns of predominantly hydrophobic peptides (Figs 4(A)–(D)). When these mixtures of hydrophobic peptides were post-treated with carboxypeptidase from ungerminated cocoa seeds, a preferential liberation of the hydrophobic amino acids Leu, Phe, Ala, Val and Tyr was observed in all the samples (Table 1). However, the relative proportions of these amino acids were rather different (Table 1). In the case of the vicilin-like globulin from cocoa seeds, almost equal amounts of Leu and Phe were liberated preferentially. The amounts of released Ala, Val and especially Tyr were considerably lower. Leu was the predominant amino acid liberated by carboxypeptidase from the peptides derived from the legumin-class (11–12 S) globulins of hazelnuts and sunflower seeds, respectively. Similar and relatively

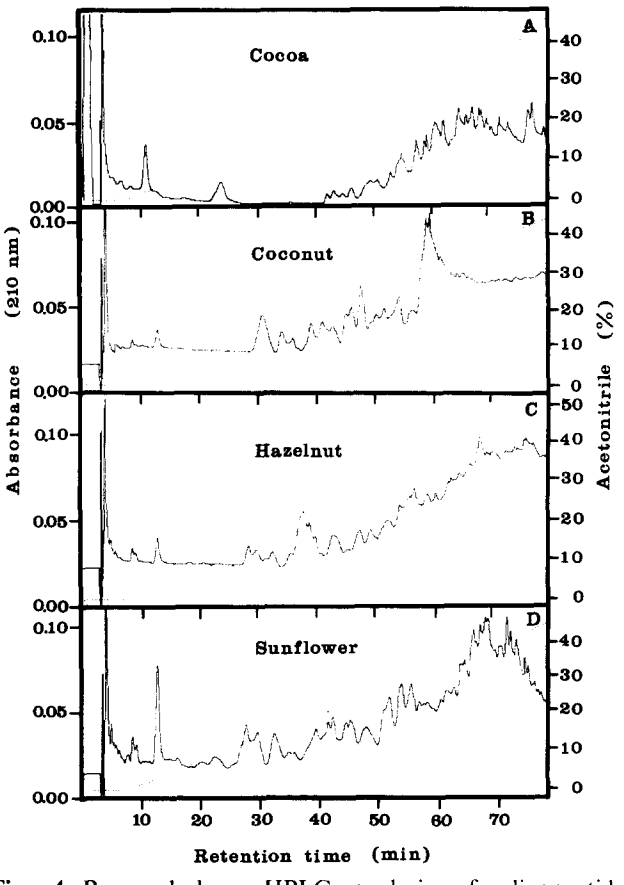


**Fig. 4.** Reversed-phase HPLC analysis of oligopeptide mixtures obtained by proteolysis of the globulins from (A) cocoa seeds, (B) coconuts, (C) hazelnuts and (D) sunflower seeds, respectively, with aspartic endoprotease from ungerminated cocoa seeds. The globulin fractions from different plants were digested with aspartic endoprotease for 16 h at pH 5·2 and 50°C and the resulting peptide mixtures analysed by reversed-phase HPLC as described in the 'Materials and Methods'.

high amounts of Ala and Phe were also released from the peptide mixtures derived from the hazelnut and the sunflower seed globulins. The amounts of released Val, however, were considerably higher in the case of the sunflower seed globulin than of the hazelnut globulin. The most striking difference from the pattern of amino acids released from the cocoa globulin peptides was observed for the coconut globulin, although both globulins belong to the vicilin class. Tyr and Ala were the predominant amino acids in the proteolysis products of coconut globulin. The amounts of released Leu and Phe were considerably lower than in the case of cocoa seed globulin.

The peptide patterns were considerably altered by carboxypeptidase treatment as revealed by reversed-phase HPLC (compare Figs 4 and 5). In contrast to the very complex patterns of hydrophobic peptides observed before carboxypeptidase treatment (Fig. 4), the carboxypeptidase-modified peptides eluted in a strongly reduced number of peaks at considerably lower acetonitrile concentrations (Figs 5(A)–(D)). Therefore, the hydrophobic peptides derived from the different globulins by degradation with aspartic endoprotease from cocoa seeds were transformed to more hydrophilic peptides by carboxypeptidase action. Different peptide patterns were obtained by successive treatments with aspartic endoprotease and carboxypeptidase of the globulins from cocoa seeds (Fig. 5(A)), coconuts (Fig. 5(B)), hazelnuts (Fig. 5 (C)) and sunflower seeds (Fig. 5(D)), respectively.

**Table 1. Free amino acids present in the proteolysis products generated *in vitro* by degradation of the globulins from cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, with aspartic endoprotease and carboxypeptidase from cocoa seeds[a]**

| Amino acid | Cocoa seed globulin | Coconut globulin | Hazelnut globulin | Sunflower seed globulin |
|---|---|---|---|---|
| Asp | 43·8 | 41·2 | 28·4 | 32·7 |
| Glu | 50·4 | 52·3 | 31·9 | 37·5 |
| Asn | 61·3 | 27·9 | 29·1 | 34·2 |
| Ser | 41·5 | 29·6 | 22·3 | 24·8 |
| Gln + His | 81·1 | 16·8 | 26·5 | 38·2 |
| Gly | 19·7 | 14·3 | 12·9 | 12·3 |
| Thr | 46·1 | 12·3 | 19·5 | 24·7 |
| Arg | 43·9 | 19·6 | 21·3 | 18·8 |
| Ala | 128·5 | 120·8 | 76·3 | 115·2 |
| Tyr | 70·6 | 136·4 | 36·9 | 49·2 |
| Trp | 12·8 | 13·7 | 15·1 | 13·6 |
| Met | 43·1 | 7·2 | 28·4 | 27·2 |
| Val | 105·7 | 27·6 | 44·8 | 97·2 |
| Phe | 179·4 | 34·2 | 85·5 | 95·0 |
| Ile | 60·9 | 12·1 | 28·5 | 52·8 |
| Leu | 167·5 | 49·7 | 155·7 | 176·4 |
| Lys | 0·9 | 4·9 | 3·3 | 1·4 |

[a] The globulin fractions prepared from cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, were digested at pH 5·2 with aspartic endoprotease and subsequently at pH 5·8 with carboxypeptidase from cocoa seeds 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.*, 1989*a*). 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.

The different proteolysis products were formulated and roasted in the presence of reducing sugars and deodorised cocoa butter and the resulting roasting aromas sensorially evaluated. As shown in Table 2, 50% of the test persons recognised cocoa or chocolate aroma in the case of the proteolysis products derived from the vicilin-class globulin of cocoa seeds. When aroma precursors isolated from fermented and air-dried cocoa seeds (Mohr *et al.*, 1971, 1976) were formulated and roasted using the same procedure, four to five out of 10 test persons recognised cocoa and chocolate aroma (data not shown; compare Voigt *et al.*, 1994*a*). No cocoa or chocolate aroma was detected by any test person when the proteolysis products of the globulins from coconuts, hazelnuts and sunflower seeds, respectively, were roasted in the presence of reducing sugars and deodorised cocoa butter (Table 2). Therefore, the particular chemical structure of the vicilin-class globulin of the cocoa seeds is the prerequisite for the proteolytic formation of the cocoa-specific aroma precursors.

**Table 2. Cocoa-specific aroma potentials of proteolysis products generated *in vitro* from the globulin fractions of cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, by the cooperative action of aspartic endoprotease and carboxypeptidase from cocoa seeds[a]**

| Protein substrate | Cocoa-specific aroma potential[b] |
|---|---|
| Cocoa seed globulin | 5/10 (50) |
| Coconut globulin | 0/10 (0) |
| Hazelnut globulin | 0/10 (0) |
| Sunflower seed globulin | 0/10 (0) |

[a] The globulin fractions prepared from cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, were subjected to proteolysis by the aspartic endoprotease at pH 5·2 and subsequently by the carboxypeptidase from cocoa seeds at pH 5·8 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 'Material 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 recognised cocoa or chocolate aroma, respectively, versus the number of test persons. As a control, aroma precursors isolated from fermented and air-dried cocoa seeds as described by Mohr *et al.* (1971, 1976) were also analysed by the same procedure. Only 4–5 out of the 10 test persons recognised cocoa and chocolate aroma in the roasting aroma of the precursors isolated from fermented cocoa seeds. The percentage of test persons who have recognised cocoa and/or chocolate aroma is shown in parentheses.

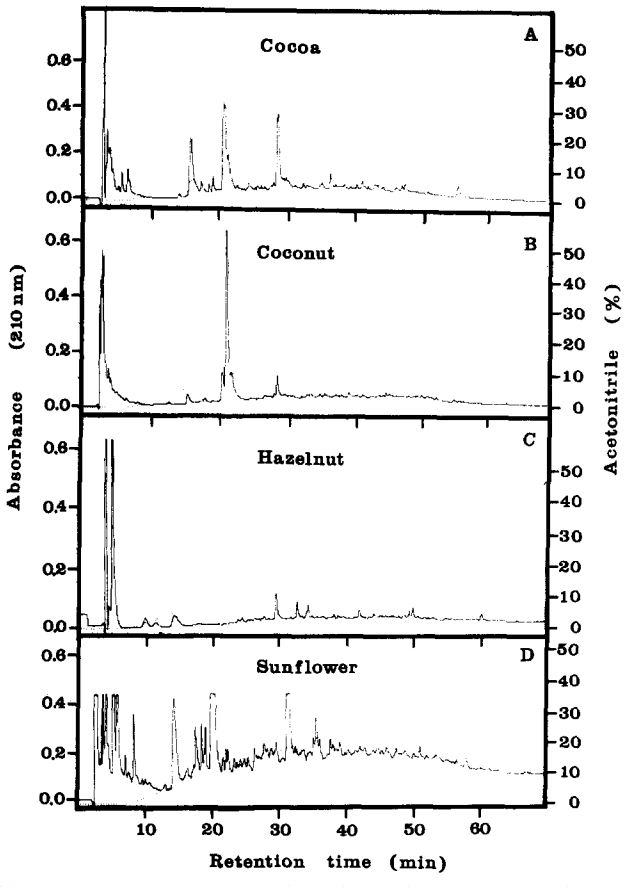


**Fig. 5.** Reversed-phase HPLC analysis of oligopeptide mixtures obtained by proteolysis with aspartic endoprotease and carboxypeptidase from cocoa seeds of the globulins isolated from (A) cocoa seeds, (B) coconuts, (C) hazelnuts and (D) sunflower seeds, respectively. The different globulins were digested with aspartic endoprotease and the resulting oligopeptide mixtures post-treated with carboxypeptidase from ungerminated cocoa seeds and subsequently analysed by reversed-phase HPLC as described in the 'Materials and Methods'.

## DISCUSSION

Oligopeptides generated during the fermentation of cocoa seeds have been found to include essential precursors of the cocoa-specific aroma components (Rohan, 1964; Mohr *et al.*, 1971, 1976; Ziegleder & Biehl, 1988). As shown by aseptic incubations of cocoa seeds, an acid-induced proteolysis of seed proteins by endogenous proteases, but not the presence of microorganisms, is required for the formation of these typical aroma precursors (Biehl *et al.*, 1985). This process is accompanied by the liberation of predominantly hydrophobic amino acids (Kirchhoff *et al.*, 1989*a,b*).

Recently, it has been shown that cocoa-specific aroma precursors can be produced *in vitro* by autolysis of acetone-dry powder prepared from unfermented cocoa seeds under appropriate conditions (Voigt *et al.* 1993*a*, 1994*a*). This finding has enabled us to study the biochemical processes involved in the formation of the aroma relevant proteolysis products. We have shown that both the aspartic endoprotease and the carboxypeptidase present in ungerminated cocoa seeds (Biehl *et al.*, 1991, 1993) are involved in this process. More or less hydrophobic peptides are generated when proteins are degraded by the aspartic endoprotease from cocoa seeds (Voigt *et al.*, 1993*a*, 1994*a*). These hydrophobic peptides have to be transformed to a mixture of hydrophilic oligopeptides and predominantly hydrophobic free amino acids to obtain cocoa-specific aroma precursors (Voigt *et al.*, 1993*a*, 1994*a*). Using purified cocoa seed proteins as substrates, it has been shown that the specific mixture of hydrophilic oligopeptides and hydrophobic free amino acids, required for the formation of the typical cocoa aroma components, is derived from the globulin of the cocoa seeds (Voigt *et al.*, 1994*b*). As shown in the present communication, cocoa-specific aroma precursors are not obtained when globulins prepared from coconuts, hazelnuts and sunflower seeds, respectively, are successively degraded by the aspartic endoprotease and the carboxypeptidase isolated from ungerminated cocoa seeds. Comparative HPLC analyses of the proteolysis derived from the globulin fractions of cocoa seeds, coconuts, hazelnuts and sunflower seeds, respectively, revealed considerable differences with respect to the patterns of free amino acids and oligopeptides. These findings indicate that the amino acid sequences of the globulins from these crops are rather different and that the formation of the cocoa-specific aroma precursors is determined by the particular chemical structure of the globulin present in the cocoa seeds.

On the basis of their amino acid sequences, subunit compositions, and the processing of the corresponding polypeptide precursors, the globular storage proteins studied so far can be assigned to two different classes: the legumin-like (11–12 S) globulins and the vicilin-like (6–8 S) globulins (Derbyshire *et al.*, 1976; Higgins, 1984; Müntz *et al.*, 1985; Borroto & Dure, 1987; Shotwell & Larkins, 1988). It has been shown that cocoa seeds exclusively contain a vicilin-class globulin (McHenry & Fritz, 1992; Spencer & Hodge, 1992, Voigt *et al.* 1993*b*). Since hazelnuts and sunflower seeds, respectively, only contain legumin-class (11–12 S) globulins (Derbyshire *et al.*, 1976; this communication), the finding that cocoa-specific aroma precursors cannot be derived from these globular storage proteins is not unexpected. As cocoa seeds exclusively contain a vicilin-class globulin (McHenry & Fritz, 1992; Spencer & Hodge, 1992; Voigt *et al.*, 1993*b*), vicilin-class (6–8 S) globulins from other crops seem to be more suitable substrates for the proteolytic formation of cocoa-specific aroma precursors than legumin-class globulins. However, no cocoa or chocolate aroma was generated upon roasting of the proteolysis products obtained by degradation of the vicilin-class globulin from coconuts with the aspartic endoprotease and the carboxypeptidase from ungerminated cocoa seeds. Therefore, we assume that the cocoa-specific aroma precursors are predominantly derived from those areas of the vicilin-class globulin of the cocoa seeds which are strongly modified during evolution of the genes encoding the vicilin-class globulins (Borroto & Dure, 1987). However, more vicilin-class globulins from different plants have to be studied as putative substrates for the proteolytic formation of the cocoa-specific aroma precursors to verify this assumption.

## ACKNOWLEDGEMENTS

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 Mr G. Gaim Marsoner and Dr A. Hugi (Jacobs-Suchard, Research et Development, Neuchatel, Switzerland) for their help with the aroma assessment. This work has been supported by a grant (AIF 8427) from the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen e.V./Forschungskreis der Ernährungsindustrie (FEI).

## REFERENCES

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., Ziegeler-Berghausen, H., Srivastava, S., Xiong, Q., Passern, D., Heinrichs, H. & Hammoor, M. (1991). Cocoa-specificity of proteolytic flavour precursors: the cocoa seed proteases. In *Proceedings of the 1991 International Cocoa Conference: Challenges in the 90s*, Kuala Lumpur, Malaysia.

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

Borroto, K. & Dure, III, L., (1987) The globulin seed storage proteins of flowering plants are derived from two ancestral genes. *Plant Mol. Biol.*, **8**, 113–31.

Derbyshire, E., Wright, D. J. & Boulter, D. (1976). Legumin and vicilin, storage proteins of legume seeds. *Phytochemistry*, **15**, 3–24.

Higgins, J. V. (1984). Synthesis and regulation of major proteins in seeds. *Ann. Rev. Plant Physiol.*, **35**, 191–221.

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**, 16–79.

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.

Macdonald, H. M., Masters, K. E., Hoddinott, C. P., Ford, G. D., Lea, A. G. H. & Pettipher, G. L. (1991). Biochemical and chemical studies on cocoa flavour precursors. *Proc. 1991 Int. Conf.: Challenges in the 90s*, Kuala Lumpur, Malaysia, (in press).

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.

Müntz, K., Bassüner, R., Lichtenfeld, C., Scholz, G. & Weber, E. (1985). Proteolytic cleavage of storage proteins during embryogenesis and germination of legume seeds. *Physiol. Vég.*, **23**, 75–94.

Quesnel, V. C. (1957). *Curing Cocoa in the Laboratory*. Rep. Cocoa Conf., Cocoa, Chocolate and Confectionary 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.

Shotwell, M. A. & Larkins, B. A. (1988). The biochemistry and molecular biology of seed storage proteins. In *The Biochemistry of Plants. A Comprehensive Treatise* (Vol. 15), ed. A. Marcus. Academic Press, New York, USA, pp. 297–345.

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. (1991). Cloning and sequencing of the cDNA encoding the major albumin of *Theobroma cacao*. *Planta* **183**, 528–35.

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.

Tai, H., McHenry, L., Fritz, P. J. & Furtek, D. B. (1991). Nucleic acid sequence of a 21 kDa cocoa seed protein with homology to the soybean trypsin inhibitor (Kunitz) family of protease inhibitors. *Plant. Mol. Biol.* **16**, 913–915.

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 formation of cocoa flavour precursors. In *Progress in Flavour Precursor Studies*, ed. P. Schreier, Allured Publishing Corp, Wheaton, 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.

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