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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

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Cocoa-specific aroma precursors were obtained when acetone-dry powder prepared from unfermented cocoa seeds was subjected to autolysis at pH 5·2. Hydrophobic free amino acids and hydrophilic peptides were preferentially generated under these conditions. When this mixture of proteolysis products was formulated and roasted in the presence of reducing sugars, cocoa and/or chocolate aroma was detected by two independent panels. Aroma precursors extracted and partially purified from fermented cocoa seeds also consisted predominantly of hydrophilic peptides and hydrophobic free amino acids. No cocoa-specific aroma precursors were obtained when the acetone-dry powder from unfermented cocoa seeds was incubated at pH 3·5. Few free amino acids were released under these conditions, but a large number of hydrophobic peptides were formed. When these hydrophobic peptides were digested with carboxypeptidase A from porcine pancreas, mixtures of hydrophilic peptides and hydrophobic free amino acids were generated, which were shown to contain cocoa-specific aroma precursors. No typical cocoa aroma was, however, obtained when synthetic mixtures of amino acids adapted to the spectrum of free amino acids present in fermented cocoa seeds (or aroma-precursor extracts) were roasted in the presence of reducing sugars. Our findings therefore indicate that the essential cocoa-specific aroma precursors are among the hydrophilic oligopeptides. Ungerminated cocoa seeds contain one predominant endoprotease (an aspartic endoprotease with a pH optimum at pH 3·5) and a carboxypeptidase activity (pH optimum: 5·8). Our findings indicate that the co-operative action of these two enzymes on cocoa-seed protein is required for the generation of the cocoa-specific aroma precursors.

INTRODUCTION

Cocoa aroma is developed on the roasting of the fermented but not of unfermented cocoa seeds (Rohan, 1964). Mohr and his co-workers have fractionated and partially characterized the cocoa-specific aroma precursors extracted from fermented cocoa seeds (Mohr *et al.*, 1971, 1976). These authors have reported that a 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). The conclusion that the cocoa-specific aroma precursors are formed during fermentation by proteolytic processes (Ziegleder & Biehl, 1988) was further corroborated by the findings that 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). Raw-

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cocoa batches with high aroma potential are generally obtained from fermentations with moderate nib acidification (pH 5·0–5·5), whereas strong acidification (pH 4·0–4·5) results in raw cocoas with low aroma potential (Biehl *et al.*, 1985). Fermentation-like incubations of 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 that are selectively degraded during seed incubations at pH 5·0–5·5 have been shown recently to be the polypeptide sub-units of a vicilin-type globulin (Spencer & Hodge, 1992; Voigt *et al.*, 1993).

The finding that there is a correlation between the formation of cocoa-specific aroma precursors, proteolysis of seed proteins, and the degree and time course of acidification during the fermentation process has 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).

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.*, 1991). 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*). The finding that, at pH 5·5, considerably higher amounts of amino acids are liberated than at pH 4·5 (Kirchhoff *et al.*, 1989*b*) may be an indication that an exopeptidase is also involved in the formation of cocoa-specific aroma precursors. On the other hand, the higher flavour potential obtained at pH 5·0–5·5 could be due to the selectivity of storage-protein degradation observed at moderate nib acidification (Biehl *et al.*, 1982). We have therefore investigated the generation of cocoa-specific aroma precursors by *in-vitro* proteolysis starting from acetone-dry powder prepared from ungerminated cocoa seeds.

MATERIALS AND METHODS

Materials

Cocoa seeds were obtained 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 (four or five 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 extracted repeatedly in a Soxhlet apparatus with 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 (AcDP)

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 5mM 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, the efficiency of polyphenol extraction was checked by heating an aliquot of the acetone-dry powder with 5M 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.

Autolysis

Acetone-dry powder from ungerminated ripe cocoa seeds (80 g each) was suspended in 8 litre of distilled water. The pH value was adjusted to 3·5 by the addition of acetic acid or to 5·2 by the addition of sodium acetate buffer (final concentration: 10mM) or to 6·8 by the addition of sodium phosphate buffer (final concentration: 5mM). The suspensions were incubated at 50°C, in a shaking water bath for 16 h. Autolysis at pH 6·8 was stopped after 8 h, the suspension adjusted to pH 5·5 by the addition of acetic acid, and further incubated at 50°C for another 8 h.

Extraction of proteolysis products

After incubation, 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 was removed under pressure at 40°C by means of a rotary evaporator. Finally, the aqueous solutions were freeze-dried.

The following yields of methanol-soluble proteolysis products were obtained from 80 g AcDP:

pH 3·5:	18–21 g
pH 5·2:	10–13 g
pH 6·8/pH 5·5:	2–3 g

Digestions with carboxypeptidases A and Y

Peptide mixtures obtained by autolysis of AcDP at pH 3·5 (5–10 g) were dissolved in 2 l of 5mM sodium phosphate (pH 7·5). After the addition of 300 units of carboxypeptidase A from porcine pancreas (53 units/mg protein; Sigma Chemie, Deisenhofen, Germany), the solution was incubated at 25°C in a shaking water bath. After 3 h, another 300 units of carboxypeptidase A were added. Incubation was stopped after 16 h. The resulting mixtures of peptides and free amino acids were freeze-dried. Digestions with carboxypeptidase Y from yeast (Hayashi *et al.*, 1973; Hayashi, 1977) were exclusively performed on an analytical scale. Peptide mixtures obtained by autolysis of AcDP at pH 3·5 (20 mg) were dissolved in 2 ml of 5mM sodium phosphate (pH 6·8). After the addition of 0·3 unit of carboxypeptidase Y from baker's yeast (100 units/mg protein; Sigma Chemie, Deisenhofen, Germany), the reaction mixtures were incubated at 25°C. Aliquots were taken after 1 and 5 h, respectively, and analysed for liberated free amino acids.

Sensory evaluation

Proteolysis products (0·75 g) or amino acids were mixed with 0·25 g glucose, 0·75 g fructose, and 0·3 g water. After the addition of 8·25 g deodorized cocoa butter, the mixtures were formulated either with mortar and pestle or by ultrasonication for 30 s by using a Branson Sonifier B12 at maximum 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 aromas generated were evaluated by sniffing analysis. Evaluations of all the roasting aromas were independently performed by a test panel (ten test persons) of the cocoa-processing industry (Jacobs-Suchard, Neuchatel, Switzerland) and by a panel (seven test persons) at the Technical University of Braunschweig (Germany). 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 guide to the evaluation of the roasting aromas. Each aroma analysis was repeated at least three times with different preparations of the same type.

HPLC analysis of peptides

Peptide mixtures were analysed by reversed-phase HPLC by using the HPLC system Gold (Beckman Instruments, San Ramon, CA, USA) equipped with an Ultrasphere ODS 5μ column (4·6 mm × 25 cm). 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.

Amino-acid analyses

The amino acids were converted into the *o*-phthalaldehyde (OPA) derivatives, separated by reversed-phase HPLC by 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).

Enzyme assays

Aspartic endoprotease

Aspartic endoprotease was isolated from cocoa seeds as described elsewhere (Biehl *et al.*, 1993). Enzyme activity was measured in McIlvaine buffer (0·2 mol/litre Na_2HPO_4 adjusted to the desired pH value by the addition of citric acid). The 1-ml reaction mixtures contained 9 mg of bovine serum albumin as substrate and 10 μg of purified aspartic protease (0·08 unit). The samples were incubated at 30°C for 1 h. The reaction was stopped by the 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 trinitro-benzenesulfonic acid method as described by Shutov *et al.* (1982).

Carboxypeptidase activity

Polyphenol-free acetone-dry powder (AcDP) was suspended in 0·02M Na_2HPO_4 adjusted to pH 6·8 by the addition of citric acid (50 mg AcDP/ml). The suspension was homogenized for 5 min at 0–3°C by using a Potter–Elvejhem homogenizer and pre-incubated in an ice bath in the presence of 10 μg/ml pepstatin A (Sigma Chemie, Deisenhofen, Germany) for 1 h to inhibit the aspartic endoprotease (Biehl *et al.*, 1991, 1993). Aliquots of the homogenates (60 μl) were added to 0·9 ml of McIlvaine buffer (0·2 M Na_2HPO_4 adjusted to the indicated pH values by the addition of citric acid). After the addition of Z-Phe-Leu (Sigma Chemie, Deisenhofen, Germany) to a final concentration of 5mM (40 μl of a 125mM stock solution in methanol), the samples were incubated for 3 h at 45°C. The reaction was stopped by the addition of 0·2 ml of 25% (w/v) trichloroacetic acid and the precipitated protein removed by centrifugation at 10 000*g* for 15 min. Finally, the released leucine was determined colorimetrically by the trinitrobenzene–sulfonic acid method as described by Shutov *et al.* (1982).

*Leucine-*p*-nitroanilide-cleaving activity*

Polyphenol-free AcDP was homogenized in 0·02M Na_2HPO_4 adjusted to pH 6·8 by the addition of citric acid (30 mg AcDP/ml). The homogenate was pre-incubated in the presence of 10 μg/ml pepstatin A (Sigma Chemie, Deisenhofen, Germany) for 1 h in an icebath to inhibit aspartic endoprotease. Aliquots (0·2 ml) of

the pre-incubated homogenates were mixed with 0·2 ml of 2mM L-leucine-*p*-nitroanilide (Sigma Chemie, Deisenhofen, Germany) and 5·6 ml of McIlvaine buffer (0·2M Na_2HPO_4 adjusted to the indicated pH values by the addition of citric acid). The reaction mixtures were incubated at 45°C for 2 h. Finally, the hydrolytic liberation of *p*-nitroaniline was determined photometrically at 400 nm (Passern, 1979; Biehl *et al.*, 1993) after removal by centrifugation of the insoluble material.

RESULTS

It has been supposed that the cocoa-specific aroma precursors are proteolysis products formed during fermentation by proteolysis of seed proteins by endogenous proteases of the cocoa seeds (Biehl *et al.*, 1982, 1985). We have, therefore, tried to produce cocoa-specific aroma precursors by *in-vitro* proteolysis. To this end, acetone-dry powder (AcDP) prepared from unfermented ripe cocoa seeds was subjected to autolysis under different pH conditions. One set of incubations was performed at pH 3·5, the optimum pH of the predominant endoprotease of ungerminated cocoa seeds (Fig. 1C; Biehl *et al.*, 1991, 1993). The second set of incubations was performed at pH 5·2 because raw cocoa with optimal aroma potential was obtained from fermentations in which nib acidification did not exceed pH 5·0 (Biehl *et al.*, 1985). It is striking that these optimal fermentation conditions correspond to the optimum pH of the carboxypeptidase of ungerminated cocoa seeds (Fig. 1B; compare also Biehl *et al.*, 1993). Since the time course of the acidification may be important for the specificity of the proteolysis, we also performed two-step incubations at pH 6·8 and pH 5·5. These pH values were selected because a leucine-*p*-nitroanilide-cleaving enzyme activity with an optimum at pH 6·8 was found in ungerminated cocoa seeds (Fig. 1A).

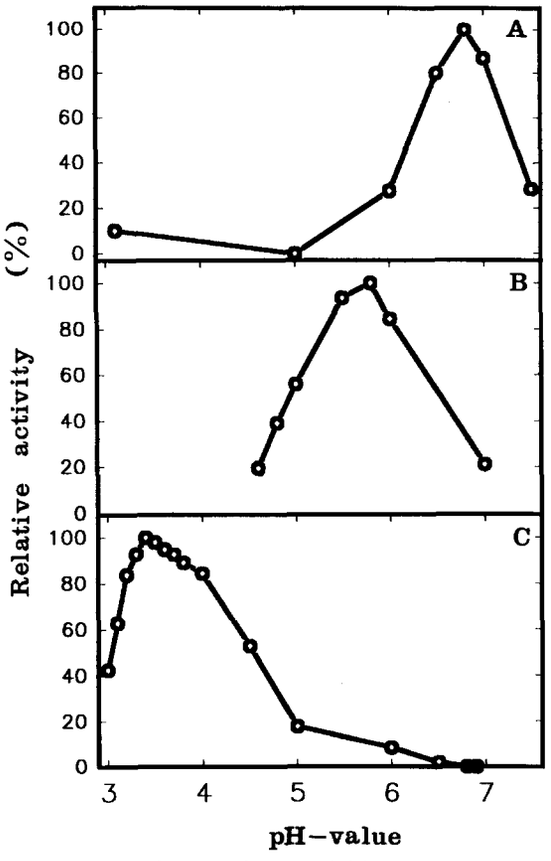

Fig. 1. pH-Dependences of the activities of the leucine-*p*-nitroanilide-cleaving enzyme (A), the carboxypeptidase (B), and the aspartic endoprotease (C) of ungerminated cocoa seeds. The pH-dependence of the aspartic endoprotease (A) was investigated on the purified enzyme by using bovine serum albumin as substrate. The other enzyme activities were determined in extracts of polyphenol-free acetone-dry powder from ungerminated cocoa seeds as described in the section headed 'Materials and Methods'.

The proteolysis products obtained were extracted with 70% (v/v) methanol and (after removal of the solvents) tested for aroma precursors (Table 1). To this end, the extracted aroma precursors were formulated and roasted in the presence of reducing sugars and deodorized cocoa butter, and the resulting aromas were evaluated by two independent test panels. Since the analysis of cocoa-aroma-precursor extracts is not an established procedure of the cocoa-processing industry, aroma precursors extracted from fermented cocoa seeds (obtained from the Ivory Coast) were used as control. When this aroma-precursor extract prepared according to Mohr and his co-workers (1971, 1976) was analysed as described above, only 30% and 43%, respectively, of

Table 1. Cocoa-specific aroma potentials of proteolysis products generated from cocoa seeds *in situ* or from isolated cocoa-seed proteins *in vitro*[a]

Source of proteolysis products	Cocoa-specific aroma potential[b] BS	N
Fermented seeds (Ivory Coast)	3/7 (43)	3/10 (30)
Autolysis of AcDP[c] at pH 5·2	3/7 (43)	4/10 (40)
Autolysis of AcDP[c] at pH 6·8 and subsequently at pH 5·5	0/7 (0)	0/10 (0)
Autolysis of AcDP[c] at pH 3·5	0/7 (0)	0/10 (0)

[a] Aroma precursors were extracted with 70% (v/v) methanol. The extracts were passed through a polyamide column to remove polyphenols.

[b] Proteolysis products were formulated and roasted in the presence of reducing sugars as described in the section headed 'Materials and Methods'. Roasting and sensory evaluation of the resulting aromas by sniffing analysis was performed independently at Braunschweig (BS) and Neuchatel (N). Values (mean values) are given as the number of test persons who have recognized cocoa and chocolate aroma, respectively, versus the number of test persons. Brackets: percentage of test persons who have recognized cocoa and/or chocolate aroma.

[c] Polyphenol-free acetone-dry powder (AcDP) prepared from unfermented cocoa seeds was subjected to autolysis at the indicated pH values as described in the section headed 'Materials and Methods'.

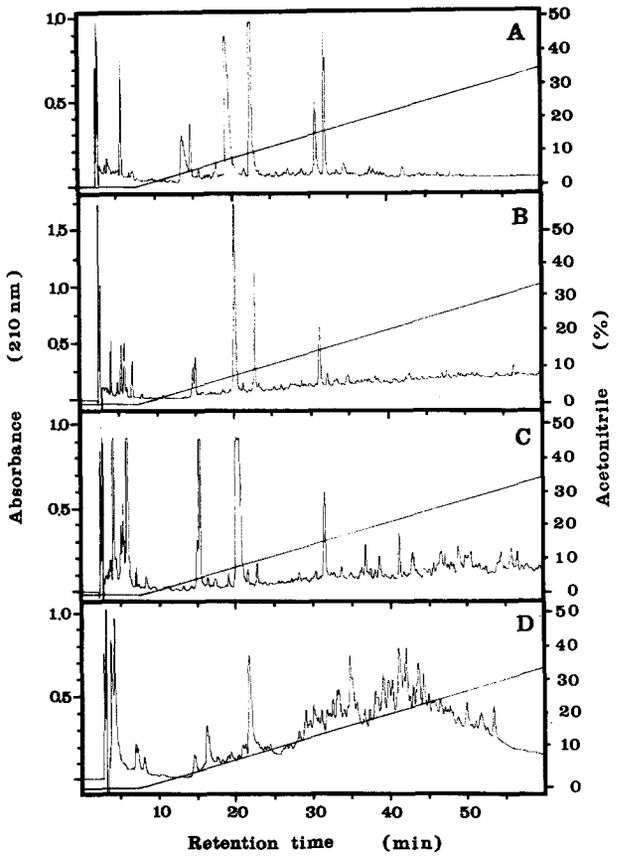

Fig. 2. Reversed-phase HPLC analysis of peptide mixtures isolated from fermented cocoa seeds (A) and *in-vitro* proteolysis obtained by autolysis of acetone-dry powder from unfermented cocoa seeds at pH 5·2 for 16 h (B), at pH 6·8 for 8 h and subsequently at pH 5·5 for another 8 h (C) and at pH 3·5 (D), respectively. Elution of the Ultrasphere ODS 5 μ column (4·6 mm × 25 cm) was performed at a flow rate of 1·0 ml/min with 0·1% (v/v) trifluoroacetic acid for 7 min followed by a linear gradient of 0 to 50% (v/v) acetonitrile containing 0·1% (v/v) acetonitrile (75 min). Elution of the peptides was monitored at 210 nm.

the test persons of both panels recognized cocoa aroma by sniffing analysis (Table 1). The results of the sniffing analyses could not be improved by modifications of the formulation and roasting conditions. Obviously, some additional flavour notes apart from the roasting aromas of the precursors are required to obtain the complete impression of 'cocoa'. On the other hand, cocoa aroma was never found by any test person when the aroma-precursor extracts were substituted by peptide mixtures of different origin (data not shown).

When polyphenol-free acetone-dry powder (AcDP) prepared from unfermented cocoa seeds was subjected to autolysis at pH 5·2 (16 h at 50°C) and subsequently extracted with 70% (v/v) aqueous methanol, an aroma precursor extract was obtained which—after roasting in the presence of reducing sugars—revealed a cocoa aroma that was recognized by 40% and 43%, respectively, of the members of both test panels (Table 1). Autolysis of AcDP at pH 3·5 or two-step autolysis at pH 6·8 and pH 5·5, however, did not result in the formation of cocoa-specific aroma precursors (Table 1).

Peptides extracted with 70% (v/v) aqueous methanol from fermented cocoa seeds or from the different autolysis products were analysed by reversed-phase HPLC (Fig. 2). The extracts from fermented cocoa seeds (Ivory Coast; standard quality) and the methanol-soluble fraction of proteolysis products obtained by autolysis of AcDP at pH 5·2 revealed very similar HPLC chromatograms (Fig. 2A,B). The predominant proportion of the peptides was eluted in a few peaks at low concentrations of acetonitrile. A similar peptide pattern was obtained by two-step autolysis of AcDP at pH 6·8 and subsequently at pH 5·5 (Fig. 2C). However, the proportions of several more hydrophobic components were considerably increased as compared with one-step autolysis at pH 5·2 (Fig. 2B). After autolysis of AcDP at pH 3·5, a highly complex pattern of predominantly hydrophobic peptides was obtained (Fig. 2D). Analysis of the free amino acids revealed that leucine, alanine, phenylalanine, and valine were the predominant free amino acids in the extracts from fermented cocoa seeds (Table 2). The same free amino acids were found to be accumulated during one-step autolysis of AcDP at pH 5·2 and during two-step autolysis at pH 6·8 and pH 5·5 (Table 2). However, the relative proportion of free alanine was considerably higher in the extracts from fermented cocoa seeds than in the autolysis products (Table 2). On the other hand, larger amounts of phenylalanine were released *in vitro* than during fermentation. These findings are in accordance with the recent reports of Kirchhoff *et al.* (1989*a,b*). The products of the AcDP autolysis at pH 3·5 contained considerably lower proportions of free amino acids than the autolysis products generated at pH 5·2 (Table 2).

Table 2. Release of amino acids[a] during proteolysis in cocoa seeds *in situ*[b] and of isolated cocoa-seed proteins *in vitro*[c]

Amino acid	Seed fermentation	Autolysis of AcDP		
		at pH 5·2	at pH 6·8/pH 5·5	at pH 3·5
Asp	29·9	35·0	38·8	40·7
Glu	110	52·6	89·9	31·6
Asn	92·9	53·6	29·7	16·1
Ser	67·2	38·6	13·4	7·20
Gln + His	48·6	80·5	105	16·2
Gly	37·7	23·7	19·8	2·63
Thr	45·8	40·6	28·7	5·93
Arg	7·67	87·5	110	22·3
Ala	207	136	116	24·2
Tyr	74·7	36·1	32·7	17·1
Trp	14·9	27·1	23·8	15·5
Met	13·3	54·9	82·9	21·5
Val	128	95·4	98·1	11·8
Phe	158	206	222	70·1
Ile	73·0	68·6	72·3	8·63
Leu	232	260	259	86·9
Lys	56·8	97·1	88·6	35·0
Total	1 398	1 394	1 430	433

[a] Values given in nmol/mg AcDP dry weight.
[b] After cocoa-seed fermentation.
[c] After autolysis of acetone-dry powder prepared from unfermented seeds.

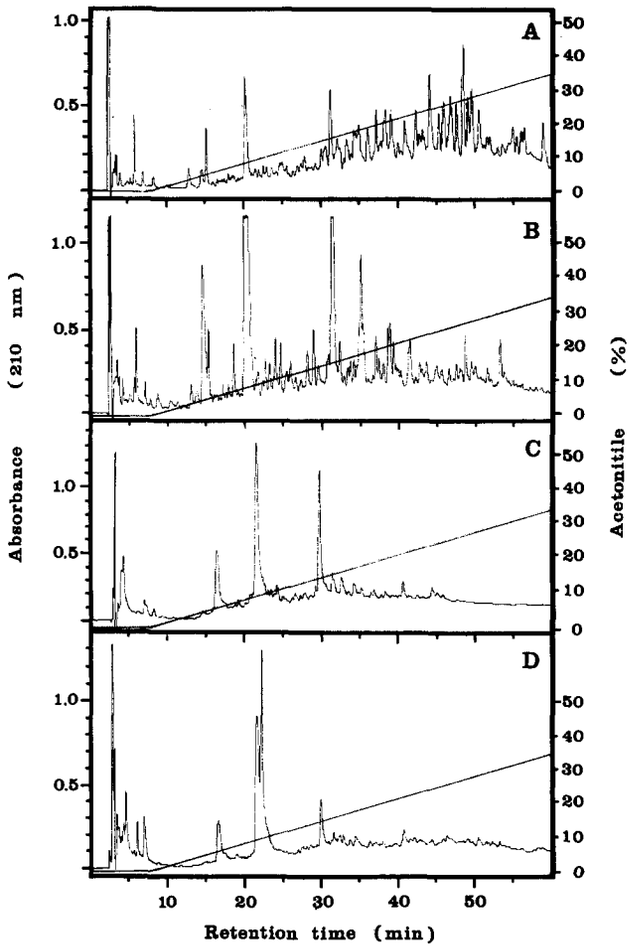

Fig. 3. Reversed-phase HPLC analysis of peptide mixtures obtained by autolysis (16 h) of acetone-dry powder from ungerminated cocoa seeds at pH 3·5 without (A) and with post-treatment with carboxypeptidase A from porcine pancreas (B,C). (A) Without treatment with carboxypeptidase A; (B) after a 1-h treatment with carboxypeptidase A; (C) after a 5-h treatment with carboxypeptidase A; (D) peptide mixture obtained by autolysis of acetone-dry powder at pH 5·2 for 16 h. Separation conditions were the same as in Fig. 1.

In conclusion, the generation of predominantly hydrophilic peptides (Fig. 2A,B) and hydrophobic free amino acids (Table 2) seems to be correlated with the formation of cocoa-specific aroma precursors (Table 1).

Since the aspartic protease is the predominant endoprotease of ungerminated cocoa seeds (Passern, 1979; Biehl & Passern, 1982; Biehl *et al.*, 1991, 1993), it is expected to be involved in the proteolytic formation of cocoa-specific aroma precursors. However, autolysis of AcDP at pH 3·5—the optimum pH of the aspartic endoprotease (Fig. 1C)—did not reveal cocoa-specific aroma precursors (Table 1). We have therefore concluded that some of the hydrophobic peptides generated by the aspartic endoprotease (Fig. 2D) may be transformed to the cocoa-specific aroma precursors by the action of an exopeptidase. The question then arose of whether the carboxypeptidase or the leucine-*p*-nitroanilide-degrading enzyme activity is involved in this process.

If the carboxypeptidase present in ungerminated cocoa seeds is responsible for the liberation of the hydrophobic amino acids found in fermented cocoa seeds and in the proteolysis products formed during autolysis of AcDP at pH 5·2 (Table 2), respectively, these hydrophobic amino acids must be located at the carboxyterminal ends of the peptides generated by the action of the aspartic endoprotease. The peptide mixture obtained after autolysis of AcDP at pH 3·5 was therefore subjected to degradation by carboxypeptidase A from porcine pancreas, which preferentially cleaves off hydrophobic carboxyterminal amino-acid residues (Neurath, 1960; Ambler, 1972). As a control, the same peptide mixture was—in parallel—degraded with carboxypeptidase Y from yeast, which cleaves off carboxyterminal amino-acid residues with a very low specificity (Hayashi *et al.*, 1973; Hayashi, 1977). In both cases, hydrophobic amino acids were preferentially liberated, and leucine and phenylalanine were the predominantly released amino acids (Table 3). Furthermore, relatively large amounts of alanine, tyrosine, and valine were liberated (Table 3). The patterns of free amino acids were rather similar to those found in fermented cocoa seeds and in the proteolysis product obtained by autolysis of AcDP at pH 5·2 (Table 2). However the relative proportions of alanine, valine, and especially tyrosine were different (compare Tables 2 and 3).

Analyses of the peptide patterns before (Fig. 3A) and after degradation with carboxypeptidase A (Fig. 3B,C) revealed that the hydrophobic peptides generated during autolysis of AcDP at pH 3·5 (Figs 2D and 3A) were transformed to considerably more hydrophilic components (Fig. 3B,C). The peptide patterns observed after a 5-h treatment with carboxypeptidase A (Fig. 3C) were very similar to the peptide patterns found after autolysis of AcDP at pH 5·2 (Fig. 3D).

Table 3. Release of amino acids by carboxypeptidases A and Y from peptides generated during pH-3·5 autolysis of cocoa-seed AcDP[a]

Amino acid	Carboxypeptidase A			Carboxypeptidase Y	
	$t = 0$	$t = 1$ h	$t = 5$ h	$t = 1$ h	$t = 5$ h
Asp	40·7	44·4	41·5	48·1	59·0
Glu	31·6	26·4	21·0	33·0	35·8
Asn	16·1	28·0	45·7	27·2	52·4
Ser	7·20	13·2	20·0	14·8	23·2
Gln + His	16·2	30·3	63·5	35·9	55·7
Gly	2·63	4·88	9·45	3·22	6·61
Thr	5·93	12·4	41·4	28·7	57·0
Arg	22·3	20·5	21·7	22·0	23·1
Ala	24·2	62·1	85·1	61·0	97·3
Tyr	17·1	54·3	96·6	53·8	99·9
Trp	15·5	17·9	27·5	16·4	24·3
Met	21·5	27·6	32·4	22·1	36·3
Val	11·8	47·3	75·1	45·6	72·5
Phe	70·1	234	252	172	248
Ile	8·63	22·9	45·7	30·4	51·3
Leu	86·9	206	276	173	273
Lys	35·0	32·1	28·6	32·1	29·2
Total	433	884	1 183	819	1 245

[a] Values given in nmol/mg AcDP dry weight.

Table 4. Cocoa-specific aroma potential of proteolysis products generated by the action of carboxypeptidase A on the pH 3·5 autolysis products of cocoa-seed acetone-dry powder

Sample	Cocoa-specific aroma potential[a] BS	N
pH-3·5 Autolysis products of cocoa-seed AcDP	0/7 (0)	0/10 (0)
pH 3·5 Autolysis products of cocoa seed AcDP treated with carboxypeptidase A	4/7 (57)	3/10 (30)
Synthetic mixture of free amino acids[b]	0/7 (0)	0/10 (0)
Phenylalanine	0/7 (0)	0/10 (0)

[a] Samples were formulated and roasted in the presence of reducing sugars as described in the section headed 'Materials and Methods'. Roasting and sensory evaluation of the resulting aromas by sniffing analyses was performed independently at Braunschweig (BS) and Neuchatel (N). Values (mean values) are given as the number of test persons who have recognized cocoa and chocolate aroma, respectively, per number of test persons (brackets: values in %).

[b] The amino-acid composition was adapted to the spectrum of free amino acids found in extracts from fermented cocoa seeds (Table 2).

When the proteolysis products were formulated and roasted in the presence of reducing sugars and deodorized cocoa butter, no cocoa-like aroma was detected by any test person in the case of hydrophobic peptides obtained by autolysis of AcDP at pH 3·5 (Table 4). However, after treatment of these peptides with carboxypeptidase A from porcine pancreas, the resulting mixtures of hydrophilic peptides (Fig. 3C) and predominantly hydrophobic free amino acids revealed a cocoa-specific aroma, which was recognized by 30 and 57%, respectively, of the members of both panels (Table 4).

To answer the question of whether the hydrophilic peptides or the specific mixture of free amino acids are the essential cocoa-specific aroma precursors, we have prepared a synthetic mixture of free amino acids whose composition was adapted to the spectrum of free amino acids found in fermented cocoa seeds and in the proteo-lysis products formed *in vitro* during autolysis of AcDP at pH 5·2 (Table 2). When this mixture of free amino acids, with leucine, phenylalanine, alanine, and valine as the major components, was roasted in the presence of reducing sugars, no test person was able to detect a cocoa or chocolate aroma in these samples (Table 4). Control experiments with phenylalanine instead of the amino-acid mixtures also gave negative results.

DISCUSSION

Several years ago, Mohr and his co-workers (1976) reported that cocoa aroma was formed when a peptide fraction isolated from fermented cocoa seeds was roasted in the presence of free amino acids and reducing sugars. These observations of Mohr *et al.* (1976) have been corroborated by our findings that cocoa-specific aroma precursors are generated during autolysis of polyphenol-free acetone-dry powder prepared from unfermented cocoa seeds (Table 1). *In-vitro* formation of cocoa-specific precursors was exclusively observed under moderate acidic conditions (pH 5·2):

(a) where considerable amounts of proteolysis products were generated and
(b) where the patterns of proteolysis products were similar to the patterns of peptides and free amino acids found in standard raw cocoas (Fig. 2; Tables 1 and 2).

Since both peptides and free amino acids are formed under these conditions, the question arises as to whether the peptides are really required for the generation of the cocoa-specific roasting aroma or whether the free amino acids liberated, during fermentation or during *in-vitro* proteolysis at pH 5·2, are responsible for the formation of the cocoa or chocolate aroma.

When a synthetic mixture of amino acids adapted to the patterns of free amino acids found in fermented cocoa seeds and in autolysis products of AcDP formed at pH 5·2, respectively, was formulated and roasted in the presence of reducing sugars, no cocoa or chocolate aroma was detected (Table 4). This finding indicates that the fraction of hydrophilic peptides generated during fermentation of cocoa seeds and during autolysis of AcDP at pH 5·2, respectively (Fig. 2A,B) contains essential cocoa-specific aroma precursors. These peptides, which are essential for the formation of the cocoa-specific aroma components during the roasting process, are obviously generated from the cocoa vicilin (Voigt *et al.*, 1993) by the co-operative action of an aspartic endoprotease and a carboxypeptidase present in ungerminated cocoa seeds. This conclusion is based on our findings that cocoa-specific aroma precursors were obtained when the mixture of hydrophobic peptides generated by the action of the aspartic endoprotease was subjected to degradation by carboxypeptidase from porcine pancreas (Table 4). The resulting proteolysis products largely consisted of hydrophilic peptides (Fig. 3C) and hydrophobic free amino acids (Table 3). These findings contribute considerably to an understanding of the fermentation process: acidification of the cocoa seeds is the prerequisite for the proteolytic digestion of the (vicilin-type) globular-storage protein (Beihl *et al.*, 1982, 1985; Voigt *et al.*, 1993) by the endogenous aspartic endoprotease of the cocoa seeds (Biehl & Passern, 1982; Biehl *et al.*, 1991, 1993). On the other hand, the resulting hydrophobic peptides have to be transformed to hydrophilic peptides and hydrophobic free amino acids by the action of the carboxypeptidase present in the cocoa seeds. Since the activity of this carboxypeptidase is rather low at pH values below 5·0 (Fig. 1B; Biehl *et al.*, 1993), a strong acidification during the fermentation process (to pH 4·5–4·0) must result in raw-cocoa batches with low aroma potential (cf. Biehl *et al.*, 1985).

The optimum fermentation conditions (acidification to pH 5·5–5·0) are dependent on the optimum pH values of the two enzymes (aspartic endoprotease and carboxypeptidase) involved in the generation of the essential, cocoa-specific aroma precursors.

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