Food Chemistry 31 (1989) 295–311

Peculiarity of the Accumulation of Free Amino Acids during Cocoa Fermentation

P.-M. Kirchhoff, B. Biehl & G. Crone

Botanisches Institut, Technische Universität, Braunschweig, FRG

(Received 18 May 1988; accepted 8 June 1988)

ABSTRACT

Based on data from several authors and from our own analyses, the composition of free amino acids (fa) in fermented cocoa seeds is compared with the amino acid composition in hydrolysates (ha) of unfermented cocoa seeds, legume seed globulins and cocoa seed storage proteins. The ha of several cocoa samples are quite uniform and do not differ significantly from the ha in legume globulins. They contain 33%:30% hydrophobic:acidic amino acids. The fa in fermented cocoa seeds are significantly different (58%:16%). The ha of a fraction enriched in cocoa storage protein (41%:26%) which is the source of free amino acids is one, though insufficient, explanation of this difference. Fermentation-like seed incubations reveal that exudation of amino acids is not responsible but free acidic amino acids are metabolized during fermentation whereas leucine, alanine, phenylalanine and tyrosine are considerably accumulated, presumably due to specific activity of endopeptidases. Cocoa flavour is considered in relation to these findings.

INTRODUCTION

Cocoa flavour is developed on roasting of fermented but not of unfermented cocoa seeds (Knapp, 1937; Rohan, 1964). Amino acids and peptides are released from proteins enzymatically during fermentation (De Witt, 1957; Biehl, 1961). They have been shown to be precursors of cocoa flavour, undergoing Maillard reactions during roasting (Mohr *et al.* 1971; Rohan, 1972). It is not known which of the numerous volatiles formed are specific to

Food Chemistry 0308-8146/89/$03·50

cocoa flavour. Several authors have tried to recognize flavour specificity of individual precursors by analyzing free amino acids in unfermented, fermented, roasted and conched cocoa (Rohan, 1964; Pinto & Chichester, 1966; Rohan & Stewart, 1966; Mohr *et al.*, 1971; Mohr *et al.*, 1976; Ziegleder & Sandmeier, 1982); however, the peculiarities of the formation of amino acids during fermentation were not considered. Rohan (1964) studied free amino acids from unfermented and fermented cocoa beans. It was attempted to relate variations in the composition in different fermented samples to differences in flavour. Rohan & Stewart (1966) estimated the losses of individual free amino acids during roasting of fermented cocoa beans and found that none of them were reduced to limiting amounts, since each amino acid was only partially lost. Pinto & Chichester (1966) also compared free amino acids in fermented cocoa before and after roasting and considered the formation during roasting of isobutyraldehyde and isovaleraldehyde from amino acids which increased during fermentation. Mohr *et al.* (1976) ascribed significance in the peak of cocoa specific flavour to the amino acids leucine, alanine, phenylalanine and tyrosine. These authors did not consider the dominance of leu, phe, tyr and ala among the free amino acids of fermented cocoa beans in previously published analyses (Pinto & Chichester, 1966; Mohr *et al.*, 1971). However, Seiki (1973), following amino acid formation during tray and heap fermentations, paid attention to this dominance since the sharp decrease of glu, leu, phe and tyr during roasting indicated their importance to the development of chocolate flavour.

In this paper, published and our own analyses of free and protein bound amino acids in unfermented and fermented cocoa seeds are compared and experiments are extended to demonstrate and to understand the preferential release of these amino acids during proteolysis from storage proteins of cocoa seeds which are rich in hydrophobic amino acids. In preceding papers (Biehl & Passern, 1982; Biehl *et al.*, 1982, 1985) we have shown that seed storage proteins are almost exclusively degraded during fermentation. Both flavour potential and protein hydrolysis were found to depend on acidity and on water uptake in the seeds during fermentation.

MATERIALS AND METHODS

Material

Cocoa seeds were from ripe, genetically undefined pods harvested as usual at the Cocoa Research Institute of Ghana, the Cocoa and Coconut Division, Hilir Perak of the Malaysian Agricultural Research and Development

Institute and from a greenhouse of the Gesamthochschule Kassel, Fachbereich Internationale Agrarwirtschaft Witzenhausen (FRG). Unfermented seeds were taken from fresh pods (in the case of Malaysian fruits ten days after harvesting). Unfermented, fermented or incubated seeds were immediately frozen and lyophilized.

Fermentations and incubations

A random sample of 2 kg was taken after five days from a normal heap fermentation in Ghana with 180 kg of fresh cocoa. Aseptic incubations of seeds without pulp (40–100 g FW) in buffer solutions in a nitrogen atmosphere under conditions equivalent to fermentation were performed as described previously (Biehl & Passern, 1982; Biehl *et al.*, 1985). Initial incubation at 40°C in sodium citrate buffer solution (pH 5·5; 35 mM; 1·5 ml/seed) was followed immediately by a second incubation at 50°C in sodium acetate buffer solution (pH 4·5; 0·2M; 5 ml/seed). During the second incubation the pH value dropped to 4·5. After incubation the media were separated from the seeds quantitatively and immediately frozen in liquid nitrogen, stored at −20°C and lyophilized to less than 3% water content.

Estimation of water content and pH value of seeds

Seeds were dried at 104°C for 24 h for estimation of water content. Undried seeds were peeled. The cotyledons were homogenized in a mortar with water (1 g/10 ml). The pH value was determined using a pH electrode.

Extraction of fat

Testae and radiculae were removed, the dry cotyledons were crushed and extracted repeatedly in a Soxhlet apparatus with petroleum spirit (bp 40–70°C). After solvent evaporation the material was powdered and extracted again for 4 to 8 h in the same manner. Finally, purin alkaloids were partially extracted with chloroform for 1·5 to 8 h in a Soxhlet apparatus.

Extraction of free amino acids

Seven hundred milligrams of the fat-free powder of fermented beans from Ghana plus 1·4 g PVPP were stirred for 1 h at 0°C in 35 ml distilled water, adjusted to pH 2·5. After centrifugation and filtration the clear solution was free of polyphenols and was used for HPLC analyses. The defatted powders from unfermented and from incubated seeds were extracted repeatedly with

aqueous acetone containing 1·25‰ thioglycollic acid. The powder was extracted three times with 80% (v/v) acetone, and five times with 70% (v/v) acetone (weight equivalent to 20 g undefatted seeds per 200 ml at 0°C, stirred for 1 h). The supernatants were separated by centrifugation (15 min; 13 000 × g). Acetone was removed from the combined supernatants in a vacuum rotary evaporator. The residual aqueous phase was made up to 420 ml with distilled water. Freeze-dried incubation media (equivalent to 40 g seed fresh weight) were homogenized at 0°C in 100 ml of 70% aqueous acetone (v/v), using an Ultraturrax tissue macerator and then filtered. After removal of acetone and repeated filtration, the volume was adjusted to 100 ml. The aqueous solutions resulting from acetone extractions were applied to columns of 10 g Polyamide SC 6 for removal of polyphenols. These solutions were used for HPLC-analyses.

Extraction and purification of proteins

Defatted powder of unfermented seeds was extracted with aqueous acetone as described above. Residual water was removed by adding pure acetone and centrifugation. Acetone was evaporated from the sediment under reduced pressure. Soluble protein was extracted from this powder. 1 g powder was homogenized in a Potter Elvehjem and stirred at 4°C for 1 h in 40 ml of a buffer solution (Tris/HCl pH 8·9; 0·268M). After filtration the resulting extract contained 2·25 mg of protein per millilitre. Protein was estimated by the method of Bradford (1976). The extract was concentrated by freeze-drying to 10 ml and was cleared by centrifugation and filtration. Five millilitres of the concentrated extract (containing 45 mg protein) was applied to a Sephadex G 150 column (diameter 5·0 cm; length 49 cm; volume 962 ml, equilibrated with 3000 ml of the same Tris/HCl buffer solution). Proteins were eluted with the same buffer solution. Fractions were separated and detected by an Isco 570 fraction collector and UV (280 nm) monitor UA-5. Six fractions were separated. The protein recovery was 100%. In Fraction 1 (17·3%) the 26 Kd and 44 Kd polypeptides of the storage proteins were enriched. For detection and quantitative determination of these polypeptides, all fractions were analyzed by SDS polyacryl amide gel electrophorosis (Biehl *et al.*, 1982). The peptide bands were stained with Coomassie Brilliant Blue, and monitored in a densitometer. An aliquot of Fraction 1 was diluted to 200 μg/ml protein and hydrolyzed with HCl.

Acid hydrolysis of proteins

1·0 ml of the protein-containing solutions plus 1·0 ml 12N HCl, containing 2·5% thioglycollic acid, were heated for 20 h at 115°C in a sealed glass tube.

HCl was removed in a rotary evaporator by repeated evaporation and addition of distilled water. The volume of the sample was adjusted to 1 ml with boric acid buffer solution for HPLC analysis.

Determination of amino acids

The amino acids were converted into *o*-phthalaldehyde (OPA) derivatives, separated by reversed phase HPLC and detected fluorometrically. Column: Shandon Hypersil ODS 5 (240 × 4·6 mm), precolumn: Shandon Hypersil ODS 10 (20 × 4·6 mm); Eluants: (A) 1600 ml sodium acetate/glacial acetic acid (pH 6·2; 50 mM), 380 ml methanol, 20 ml tetrahydrofuran; (B) 200 ml sodium acetate/glacial acetic acid (pH 6·2; 50 mM), 800 ml methanol; flow rate: 1·3 ml/min. The column was equilibrated with eluant A. Gradient (A + B = 100% v/v): (1) 2 min 100% A, (2) 8 min 100 to 95% A, (3) 2 min 95 to 85% A, (4) 4 min constant 85% A, (5) 1 min, 50 s 85 to 77% A, (6) 6 min, 10 s 77 to 50% A, (7) 10 min constant 50% A, (8) 10 min 50 to 0% A, (9) 10 min constant 0% A. Detector: Hitachi Fluorescence Photometer F-3000, equipped with a micro flow cuvette (volume 18 μl, $d = 10$ mm); excitation at 334 nm, emission at 425 nm; volume of the sample loop: 20 μl. Transformation of amino acids into *o*-phthalaldehyde derivatives: 50 μl sample of solution was freeze-dried and redissolved in 1000 μl buffer solution (200 mM boric acid in distilled water adjusted to pH 9·5 with KOH soln.) to which 500 μl reagent were added (50 mg *o*-phthalaldehyde plus 1·25 ml absol. methanol plus 50 μl 2-mercaptoethanol plus 11·2 ml borate buffer solution). Twenty microlitres of the reaction mixture were injected and the reaction was stopped after exactly 2 min by passing the eluant into the column and at the same time initiating the separation. The concentrations of individual amino acids were calculated from chromatograms of a mixture containing 50 pmol/μl of each amino acid to be determined. The standard deviation was equal to $\pm$2·2% except of glutamic acid ($\pm$2·7%), arginine ($\pm$4·4%), glycine ($\pm$4·7%) and lysine ($\pm$13%).

RESULTS

Protein bound amino acids

In Table 1 data of analyses on amino acid composition in acidic hydrolysates of unfermented cocoa seeds and the seeds of one legume are shown together for comparison. In Table 1, A, the mean values and the resulting standard deviations are calculated from amino acid determinations of sixteen genetically different cocoa seed samples, published by Timbie & Keeney (1980) and Zak & Keeney (1976).

TABLE 1

Composition of Amino Acids in Acidic Hydrolysates of Unfermented Cocoa Seed from Different Origins and of Legume Seeds

	Relative amounts of amino acids (mol%)				
	Cocoa				*Soya*[4]
Origin of seeds	*A* *16 different origins*[1,2]	*B* *West Africa*[3]	C_1	C_2 *Malaysia*	
Preparation	*Total seed hydrolysate*		*Soluble protein, acid hydrolysate*	*From fraction C_1 enriched in storage protein, acid hydrolysate*	*11S-fraction of seed globulins, acid hydrolysate*
Amino acids	M ± σ				M ± σ
Acidic	31·51	30·32	30·38	25·95	34·08
Asp + Asn	15·38 ± 2·23	12·00	12·40	10·84	13·41 ± 1·03
Glu + Gln	16·13 ± 1·06	18·32[a]	17·98	15·11	20·67 ± 2·50[a]
Hydrophobic	33·53	33·00	34·08	41·31	32·28
Leu	7·26 ± 0·27	7·52	6·74	7·32	7·50 ± 0·60
Ala	7·46 ± 0·44	7·49	7·50	9·03[a]	6·13 ± 0·47[a]
Phe	4·59 ± 0·19	4·82	4·52[a]	7·24[a]	4·74 ± 0·53
Tyr	2·70 ± 0·18	2·62	4·02	5·12[a]	3·02 ± 0·21
Val	7·38 ± 0·76	6·81	7·38	8·72	5·80 ± 0·55
Ile	4·14 ± 0·27	3·74	3·92	3·88	5·10 ± 0·56[a]

Others	34·96	36·65	35·54	32·74	33·64
Lys	6·11 ± 0·62	6·58	3·87	2·92[a]	4·41 ± 0·43[a]
Arg	5·43 ± 0·45	5·69	6·86	6·74[a]	6·09 ± 0·53
His	1·92 ± 0·20	1·79	1·32	1·48[a]	1·99 ± 0·11
Thr	5·10 ± 0·35	5·11	5·24	5·26	4·61 ± 0·76
Ser	6·70 ± 0·55	7·75	7·82	8·64[a]	7·18 ± 1·80
Gly	8·68 ± 0·76	8·81	9·35	6·19[a]	7·92 ± 0·12
Met	1·02 ± 0·16	0·92	1·08	1·51[a]	1·44 ± 0·28[a]
Total	100·00	99·97	100·00	100·00	100·00

A. B: total HCl-hydrolysates of unfermented cocoa seeds. A: mean values (M) and the resulting standard deviation (σ) were calculated from data of 16 different varieties and clones, published by Timbie & Keeney (1980) and Zak & Keeney (1976).

B. Data published by Biehl (1967). C_1, C_2: unfermented Malaysian cocoa seeds; C_1: acetone dry powder was prepared, soluble proteins were extracted with Tris/HCl-buffer as described in the section headed 'Extraction and Purification of Proteins', and were hydrolyzed in HCl. C_2: A fraction enriched in storage proteins as defined by Biehl *et al.* (1982) was separated from the buffer extract (C_1) by column chromatography as described in the above-mentioned section and was hydrolyzed. This fraction contained 73·3% of storage proteins (44 and 26 Kd) (Biehl *et al.*, 1982) compared to 19·2% in the buffer extract C_1. Soya (*Glycine max*): From several amino acid analyses of seed globulins of different Leguminosae species published by Derbyshire *et al.* (1976), those of Soya are shown. Amino acids were determined in the acid hydrolysate of the 11S-fraction from the NaCl-extracts of globulins. The mean values from data of three different analyses and the resulting standard deviations were calculated. The method of Moore & Stein (1951) was used for amino acid determination in A, B and in soya proteins. HPLC was used in the case of C. Mol % of the total amino acids listed in the table.

[a] Values differing by twice the standard deviation or more from the mean values in A. The group of hydrophobic amino acids includes tyrosine to facilitate comparison.

[1] Timbie & Keeney (1980).

[2] Zak & Keeney (1976).

[3] Biehl (1967).

[4] Derbyshire *et al.* (1976).

TABLE 2
Free Amino Acids in Fermented Cocoa Cotyledons

	Relative composition of amino acids (mol %)					
Origin of seeds	*D*[1] (*Bahia*)	*E*[2] (*Ghana*)	*F* (*Ghana*)	*G* (*Witzenhausen*)	*H* (*Malaysia*)	$M \pm \sigma$ (*D–H*)
Preparation	*Hot water extract of fat containing cotyledons*	*Extract with methanol–water of the defatted cotyledons*	*Extract with acetone–water mixtures of the defatted cotyledons*			
	Normally fermented seeds			*Incubated seeds*		
Amino acids						
Acidic[a]	*13·1*	*14·9*	*15·9*	*17·0*	*20·5*	*16·3 ± 2·8*
Asp/Asn	4·0	3·9	8·2	9·5	9·9	7·1 ± 2·9
Glu/Gln plus His[a]	9·1	11·0	7·7	7·5	10·6	9·2 ± 1·6
Hydrophobic	*59·1*	*59·3*	*58·3*	*55·3*	*58·6*	*58·1 ± 1·6*
Leu	16·2	15·8	17·4	17·7	16·4	16·7 ± 0·8
Ala	13·7	13·1	14·4	9·8	13·6	12·9 ± 1·8
Phe	10·9	11·4	9·1	11·3	11·3	10·8 ± 1·0
Tyr	5·8	6·5	4·0	7·2	9·1	6·5 ± 1·9
Val	7·5	7·8	8·6	6·2	5·0	7·0 ± 1·4
Ile	5·0	4·7	4·8	3·1	3·2	4·2 ± 0·9

Others[a]	*28·3*	*25·9*	*25·8*	*27·6*	*21·1*	*25·7 ± 2·8*
Lys	5·9	4·1	7·0	7·2	3·0	5·4 ± 1·8
Arg	6·2	3·6	4·0	9·1	6·9	6·0 ± 2·2
His[a]	—	—	—	—	—	—
Thr	6·5	5·9	4·0	2·8	2·4	4·3 ± 1·8
Ser	5·4	9·8	5·1	4·0	4·1	5·7 ± 2·4
Gly	3·4	2·2	3·2	1·9	2·6	2·7 ± 0·6
Met	0·9	0·3	2·5	2·6	2·1	1·7 ± 1·0
Total	100·5	100·1	100·0	99·9	100·2	100·1
Total amino acids (μmol/g DW)[b]	26·0	30·7	38·2	35·6	25·0	

The amino acid compositions in normally fermented (D, E, F) and in fermentation-like incubated (G, H) cocoa seeds are shown. Additionally the mean values and the resulting standard deviation (D–H) are given. D, E: data taken from the literature (D: Pinto & Chichester, 1966, E: Mohr *et al.*, 1971). These samples were air-dried as usual after fermentation. F: A random sample was taken from a heap fermentation in Ghana, but the seeds were lyophilized promptly after five days of fermentation. G, H: unfermented seeds from ripe pods were incubated in buffer solutions similar to fermentation as described in the section headed 'Extraction and Purification of Proteins' and were freeze-dried. For amino acid analyses, ion-exchange column chromatography (Moore and Stein, 1951) (D, E) or HPLC (F, G, H) were used, respectively. Mol% refer to the sum of all specified amino acids (= 100%); μmol/g refer to the nondefatted dry nibs.

[a] In contrast to analyses using the method of Moore and Stein (1951) (D, E). The HPLC-method used (F, G, H) did not allow the separation of histidine from glutamine. To allow comparison, the values published in D (2·1 mol% histidine) and in E (1·2 mol% histidine) were included in 'glu/gln plus his'.

[b] The total amount of amino acids (μmol/g DW) as given by Pinto & Chichester (1966) was multiplied by 0·108. This factor is derived from experimental methods described in this publication.

[1] Pinto & Chichester (1966).

[2] Mohr *et al.* (1971).

In all these samples, the amino acid compositions are quite uniform. Aspartic acid, glutamic acid and their amides dominate, followed by glycine. Corresponding analysis of West African cocoa (Biehl, 1967; Table 1, B) and also one of a buffer extract from Malaysian cocoa seeds (Table 1, C_1), which contained only 30–40% of the total seed protein, produced almost identical results. This uniform composition of total (free and bound) amino acids in various samples of unfermented seeds significantly differs from the composition of free amino acids in fermented cocoa seeds (Table 2), which have a very high proportion of hydrophobic, and a low proportion of acidic, amino acids.

The total amino acid composition of cocoa (Table 1, A, B) is very similar to that of a legume seed globulin (Table 1, *Glycine max*, taken from the literature; Derbyshire *et al.*, 1976). Notwithstanding some differences, the composition in cocoa is almost within the range of standard deviations found in seed analyses of several legume species as published by Derbyshire *et al.* (1976) and Dalling (1986). In cocoa, the proportions of hydrophobic and acidic amino acids are somewhat higher and lower, respectively. Thus, this comparison does not indicate any genetic peculiarities specific to cocoa seed proteins, which might otherwise be held responsible for the formation of high amounts of free hydrophobic amino acids during cocoa fermentation.

A significantly higher proportion of hydrophobic amino acids was found in a fraction which was enriched in storage proteins (Table 1, C_2 compared to A, B and C_1). Two polypeptides have been characterized by SDS-PAGE to be the main components of cocoa seed storage proteins (Biehl *et al.*, 1982). A fraction, enriched in these storage protein components was separated from the seed buffer-extract (Table 1, C_1). The total hydrolysate of this fraction contained significantly more alanine, phenylalanine and tyrosine and less acidic amino acids.

Release of free amino acids during cocoa seed fermentation

The total amounts of free amino acids and oligopeptides which are formed and accumulated in the seeds during fermentation are not constant (Rohan, 1964; Biehl *et al.*, 1985) but depend on the nib-pH during the stage of proteolysis (Biehl *et al.*, 1985). However, there is a distinct conformity in the composition of free amino acids in the fermented cocoa seeds from different origins, as evidenced by several authors (Table 2). Furthermore, there is no significant difference between normally fermented and incubated seeds. It is striking that the composition of free amino acids after fermentation (Table 2) differs strongly from that in total hydrolysates of proteins from which they are released (Table 1). The high percentage of the free hydrophobic amino

acids (leu, ala, phe) plus tyr (47%) compared to only 22% in the hydrolysate is significant, as also is the low percentage of the acidic amino acids and their amides plus glycine (19%) compared to 40% in the hydrolysate. Although the purified storage protein (Table 1, C_2) contains more hydrophobic and less acidic amino acids than the total seed hydrolysates (Table 1, A, B, C_1), the proportions of free hydrophobic or acidic amino acids are significantly higher or lower, respectively, than in the hydrolysate of the storage protein fraction. Thus, during fermentation,

TABLE 3
Distribution of Free Amino Acids from Seeds and from Seed Exudates after Fermentation-Like Seed Incubation

	Seeds		*Exudate*		*Seeds + exudate*	
	(*μmol/g*)	(*mol %*)	(*μmol/g*)	(*mol %*)	(*μmol/g*)	(*mol %*)
Amino acids						
Acidic[a]	*5·05*	*20·2*	*2·11*	*12·0*	*7·16*	*16·8*
Asp	0·58	2·3	0·18	1·1	0·76	1·8
Asn	1·86	7·4	1·03	5·8	2·89	6·8
Glu	2·07	8·3	0·52	3·0	2·59	6·1
Gln + His[a]	0·54	2·2	0·38	2·1	0·92	2·2
Hydrophobic	*14·43*	*57·7*	*12·68*	*72·1*	*27·11*	*63·6*
Leu	4·04	16·2	3·73	21·2	7·77	18·3
Ala	3·35	13·4	2·65	15·1	6·00	14·1
Phe	2·79	11·2	2·57	14·6	5·36	12·6
Tyr	2·24	8·9	1·86	10·6	4·10	9·6
Val	1·22	4·9	1·10	6·2	2·32	5·4
Ile	0·79	3·2	0·77	4·4	1·56	3·7
Others	*5·52*	*22·1*	*2·81*	*15·9*	*8·33*	*19·6*
Lys	0·74	3·0	0·40	2·3	1·14	2·7
Arg	1·69	6·8	0·53	3·0	2·22	5·2
His[a]	—	—	—	—	—	—
Thr	0·60	2·4	0·35	2·0	0·95	2·2
Ser	1·00	4·0	0·43	2·4	1·43	3·4
Gly	0·65	2·6	0·36	2·0	1·01	2·4
Meth	0·53	2·1	0·44	2·5	0·97	2·3
Try	0·31	1·3	0·30	1·7	0·61	1·4
Total	25·00	100·0	17·60	100·0	42·60	100·0

After anaerobic, acidic seed incubation of Malaysian cocoa seeds (sample H, compare Table 2) the seeds and the medium were quantitatively separated. The amino acids in the acetone/water extracts of the freeze-dried, defatted samples were estimated by HPLC. μmol/g refer to the dry weight of the seeds before fat extraction.
[a] See footnote to Table 2.

TABLE 4
Free Amino Acids in Unfermented Seeds and Changes during Fermentation-Like Incubation

Origin of seed	*Composition of free amino acids*							
	G (*Witzenhausen*)		*H* (*Malaysia*)					
	Seeds, unfermented		*a* *Seeds, unfermented*		*b* *Seeds + medium after incubation*		*c* (= *b*–*a*)	
	(*μmol/g*)	(*mol %*)	(*μmol/g*)	(*mol %*)	(*μmol/g*)	(*mol %*)	(*μmol/g*)	(*mol %*)
Amino acids								
Acidic[a]	*7·87*	*58·6*	*11·24*	*74·9*	*6·43*	*16·8*	*−4·81*	*−20·7*
Asp	3·37	25·1	3·26	21·7	0·69	1·8	−2·57	−11·0
Asn	2·52	18·8	3·35	22·3	2·59	6·8	−0·76	−3·3
Glu	1·42	10·6	3·89	25·9	2·33	6·1	−1·56	−6·7
Gln + His[a]	0·56	4·2	0·74	4·9	0·82	2·1	0·08	0·3
Hydrophobic	*3·76*	*28·0*	*2·70*	*18·1*	*24·39*	*63·7*	*21·70*	*93·0*
Leu	0·79	5·9	0·00	0·0	6·99	18·3	6·99	30·0

Ala	0·88	6·6	1·15	7·7	5·39	14·1	4·25	18·2
Phe	0·72	5·4	0·22	1·5	4·82	12·6	4·60	19·7
Tyr	0·36	2·7	0·92	6·1	3·69	9·6	2·77	11·9
Val	0·56	4·2	0·19	1·3	2·09	5·4	1·90	8·1
Ile	0·45	3·4	0·22	1·5	1·41	3·7	1·19	5·1
Others	*1·80*	*13·4*	*1·06*	*7·1*	*7·50*	*19·6*	*6·43*	*27·6*
Lys	0·32	2·4	0·00	0·0	1·03	2·7	1·03	4·4
Arg	0·50	3·7	0·22	1·5	2·01	5·2	1·78	7·7
His[a]	—	—	—	—	—	—	—	—
Thr	0·25	1·9	0·08	0·5	0·85	2·2	0·77	3·3
Ser	0·55	4·1	0·56	3·7	1·29	3·4	0·73	3·1
Gly	0·08	0·6	0·20	1·3	0·90	2·4	0·70	3·0
Met	0·05	0·4	0·00	0·0	0·87	2·3	0·87	3·7
Try	0·05	0·4	0·00	0·0	0·55	1·4	0·55	2·4
Total	13·43	100·0	15·00	99·9	38·32	100·1	23·32	99·9

Acetone/water extracts of freeze-dried sample were analyzed by HPLC. Ha, b, c are from one incubation experiment (as in Table 3). Hc: The difference b–a gives the change in free amino acids during incubation, including those exuded into the medium. In contrast to Table 3, μmol/g do not refer to the dry weight of the incubated seeds but to the dry weight of the unfermented seeds before incubation in order to avoid errors of calculation due to dry weight loss by exudation.

[a] See footnote to Table 2.

amino acids are not released unspecifically corresponding to their statistical distribution in the total seed protein, and the composition of free amino acids does not merely reflect the amino acid composition of the storage protein.

Exudation from the seeds of free amino acids during fermentation

During normal fermentations considerable amounts of especially soluble compounds are exuded from the seeds into the surrounding pulp. Up to 33% of the total nitrogen of the seeds is lost (Birch, 1941). Thus, a preferential exudation of some free amino acids may explain the characteristic composition of the residual ones in the fermented seeds. The amount of individual amino acids lost during normal fermentation cannot be estimated. During fermentation-like incubations of seeds, the extent of exudation of soluble nitrogenous compounds is similar to that in the course of normal fermentation and can be determined quantitatively (Biehl & Passern, 1982). Incubations were performed under conditions allowing biochemical processes in the seeds to run as in normal fermentation. Subsequently seeds and media (containing exuded compounds) were separated quantitatively for analyses. The results are shown in Table 3. The amounts of free amino acids in the seeds are not different from those found in normally fermented cocoa seeds. Relatively high amounts are lost by exudation (see also Biehl & Passern, 1982). The percentage loss is not uniform for all amino acids: aspartic and glutamic acid and arginine were retained in the seeds more strongly than were leucine, alanine, phenylalanine, tyrosine and valine. Besides glutamine, these hydrophobic amino acids, which predominate in the seeds, are preferentially lost. Consequently, the dominance of hydrophobic amino acids (and the corresponding paucity in acidic amino acids) is more pronounced in the exudate and in the total of exudate plus seeds, compared to seeds alone. It can thus be concluded that exudation is not responsible for the characteristic composition of free amino acids in fermented cocoa seeds.

Free amino acids in unfermented seeds and their increase during fermentation-like seed incubation

Free amino acids in fermented seeds originate from those present in the unfermented seed and from proteolysis during fermentation. Although the relative amounts of individual amino acids differ considerably in unfermented seeds of different origin (Rohan, 1964), the acidic amino acids and their amides predominate (Table 4, G, Ha). During incubation their absolute amount (Table 4, Hb: 6·43 μmol/g) is significantly decreased

through metabolization (Table 4, Hc). The remaining amino acids are increased in amount due to proteolysis. However, the significant relative increase in free hydrophobic amino acids is not due merely to the loss of the acidic amino acids: the hydrophobic amino acids constitute about 70 to 80% of the non acidic free amino acids but only about 50% of the non acidic ones in seed hydrolysates (compare Tables 1, 2, 3 and 4).

DISCUSSION

The significant difference in composition of the free amino acids released during fermentation compared with the composition in total seed hydrolysates (reflecting predominantly the protein-bound amino acids) is a striking feature of cocoa fermentation. A corresponding amino acid composition should be expected from random protein hydrolysis after seed death.

There is no indication of exceptionally high amounts of hydrophobic and low amounts of acidic amino acids in the cocoa seed protein compared to legume seed globulins (Derbyshire *et al.*, 1976; Dalling, 1986). A random proteolysis of the total seed protein would not cause the characteristic increase of hydrophobic and decrease of acidic amino acids during fermentation as documented in Tables 1 to 4.

The cocoa seed storage protein is the exclusive, or at least predominant, source of amino acids released enzymatically during fermentation (Biehl *et al.*, 1982). Since the partially purified storage protein reveals increased amounts of hydrophobic and decreased proportions of acidic amino acids compared to the crude protein extract (Table 1, C_1, C_2), its random proteolysis would contribute at least in part to the particular composition of free amino acids in fermented seeds. Alanine, phenylalanine and tyrosine, which besides leucine dominate among the free amino acids in fermented seeds, are enriched in the cocoa storage protein in contrast to legume storage protein (Table 1). However, the high relative amount of free hydrophobic amino acids in fermented seeds (about 60%, Tables 2 and 3) cannot be explained satisfactorily in this way.

It has been indicated already that the considerable amount of amino acids exuded from the seeds during fermentation is not responsible for the characteristic composition of free amino acids in the seeds after fermentation, but two other processes are assumed to play major roles as explained below.

First, the significant loss of acidic amino acids, in spite of considerable proteolysis (Table 4), contributes to the strong increase in the relative amount of hydrophobic amino acids, the absolute amount of which is

especially increased. This degradation is not a peculiarity of seed incubation but applies to normal fermentation as well (Seiki, 1973). It is not known whether or not other amino acids, besides the acidic ones, are lost, since any decrease may be compensated by their proteolytic formation. Amino acid degradation may take place prior to the death of the seeds. Microorganisms are unlikely to be involved during aseptic anaerobic seed incubation but their contribution during fermentation cannot be ruled out.

Secondly, specific endopeptidases of the seeds may split the storage protein, preferentially near to hydrophobic sections of the polypeptide chains, allowing a limited action of exopeptidases to release predominantly hydrophobic amino acids before cessation of proteolysis. High proteolytic activity is found shortly after the death of the seeds and greatly decreases during the progress of fermentation (Forsyth *et al.*, 1958). Thus, amino acids are released only in limited amounts, the main product of proteolysis being peptides (Biehl & Passern, 1982; Biehl *et al.*, 1985).

It is noteworthy that especially the amino acids leucine, alanine, phenylalanine and tyrosine, which are thought by some workers to contribute to the formation of cocoa flavour (Seiki, 1973; Mohr *et al.*, 1976), accumulate to a far greater extent than other amino acids during cocoa fermentation. These amino acids are especially reactive during roasting. Seiki (1973) found that glu, leu, tyr and phe decreased sharply during roasting. Also from the analytical data of Pinto & Chichester (1966), Mohr *et al.* (1971) and Ziegleder & Sandmeier (1982) it can be calculated that the absolute amounts of leu, ala, phe and tyr, consumed during roasting and conching, are far higher than the amounts of other amino acids.

ACKNOWLEDGEMENT

This work was supported by the Arbeitsgemeinschaft Industrieller Forschungsvereinigungen, Forschungskreis der Ernährungsindustrie, FRG, under No. 6535.

REFERENCES

Biehl, B. (1961). Untersuchungen über Veränderungen der Proteine und Aminosäuren in den Kakaokernen während der Fermentation. *Gordian*, **61**(1444), 14–20.

Biehl, B. (1967). Proteinhydrolyse während der Kakaofermentation in Abhängigkeit von Wechselwirkungen mit Polyphenolen unter anaeroben und aeroben Bedingungen. *Z. Lebensm. Unters.-Forsch.*, **133**, 145–58.

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

Biehl, B., Wewetzer, Chr. & 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 Agri.*, **36**, 583–98.

Birch, H. F. (1941). Changes in the nitrogenous components of *Forastero cacao* during fermentation. Tenth Ann. Rep. on Cacao Res., Imp. Coll. Trop. Agric, Trinidad, pp. 22–33.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem.*, **72**, 248–54.

Dalling, M. J. (1986). *Plant Proteolytic Enzymes.* CRC Press, Boca Raton, Florida, USA.

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

De Witt, K. W. (1957). Nitrogen metabolism in fermenting cacao. Ann. Rep. on Cacao Res. 1955–1956, Imp. Coll. Trop. Agric., Trinidad, pp. 54–57.

Forsyth, W. G. C., Quesnel, V. C. & Roberts, J. B. (1958). The interaction of polyphenols and proteins during cocoa curing. *J. Sci. Food Agric.*, **9**, 181–4.

Knapp, A. W. (1937). *Cacao Fermentation.* John Bale Sons & Curnow, London.

Mohr, W., Röhrle, M. & Severin Th. (1971). Über die Bildung des Kakaoaromas aus seinen Vorstufen. *Fette, Seifen, Anstrichmittel*, **8**, 515–21.

Mohr, W., Landschreiber, E. & Severin, Th. (1976). Zur Spezifität des Kakaoaromas. *Fette, Seifen, Anstrichmittel*, **2**, 88–95.

Moore, S. & Stein, W. H. (1951). Chromatography of amino acids on sulphonated polystyrenes resins. *J. Biol. Chem.*, **192**, 663–681.

Pinto, A. & Chichester, C. O. (1966). Changes in the content of free amino acids during roasting of cocoa beans. *J. Food Sci*, **31**, 726–32.

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

Rohan, T. A. (1972). Aromas in Food processing. *Dechema Monographie*, **70**, 301–21, Verlag Chemie Weinheim.

Rohan, T. A. & Stewart, T. (1966). The precursors of chocolate aroma: changes in the free amino acids during roasting of cocoa beans. *J. Food Sci.*, **31**, 202–5.

Seiki, K. (1973). Chemical changes during cocoa fermentation using the tray method in Nigeria. *Rev. Int. Choc.*, **28**, 38–42.

Timbie, D. J., Keeney, P. G. (1980). Comparison of several types of cocoa beans relative to fractionated protein components. *J. Agric. Food Chem.*, **28**, 472–83.

Zak, D. L., Keeney, P. G. (1976). Extraction and fractionation of cocoa proteins as applied to several varieties of cocoa beans. *J. Agric. Food Chem.*, **24**, 479–83.

Ziegleder, G., Sandmeier, D. (1982). Chemische Veränderungen beim Conchieren milchfreier Schokoladenmassen. *Dtsch. Lebensmittelrundschau*, **78**, 315–18.