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Kinetics of the Formation of Free Amino Acids in Cocoa Seeds during Fermentation

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ABSTRACT

The predominance of leucine, phenylalanine, alanine and tyrosine among the free amino acids released from proteins during fermentation of cocoa seeds was further studied by following the kinetics of amino acid turnover during fermentation-like seed incubation and acetone dry-powder incubation by HPLC-analyses. Free acidic amino acids of the unfermented seeds disappear before onset of post-mortem proteolysis in a premortem phase of seed incubation. During subsequent post-mortem proteolysis in whole seeds (incubated at 50°C in acetic acid/NaOH (200 mM; pH 4·5)) leucine, phenylalanine, alanine and tyrosine are predominantly released. The initial high rate of amino acid formation decreases rapidly and their relative proportions change continually. With acetone dry-powder incubations it is shown that a decreasing nib pH affects the amino acid composition but does not explain these changes, especially not the strongly decreasing ratio of (ala + tyr):(leu + phe). Since vacuolar storage proteins are degraded earlier than other seed proteins, it is assumed that this change in substrate proteins is responsible.

INTRODUCTION

The predominant accumulation of leucine, alanine, phenylalanine and tyrosine among the amino acids liberated during cocoa fermentation and the significant degradation of acidic amino acids are assumed to be important for the formation of cocoa flavour (Kirchhoff *et al.*, 1989). This

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peculiarity of proteolysis is found during fermentation-like seed incubation (Kirchhoff *et al.*, 1989) as well as during normal fermentation (Seiki, 1973).

Seiki (1973), following the formation of free amino acids in cocoa seeds during heap and tray fermentation in Nigeria, found the strongest increase during the second day. The data suggest that free glutamic acid, in contrast, is lost during the first day. De Witt (1957) and Forsyth *et al.* (1958) have shown that proteolytic activity in cocoa seeds during fermentation is highest during the second and the third days. Furthermore, the data of Seiki (1973) reveal that there is no uniform breakdown of proteins which would cause release of amino acids in constant proportions at any stage. Ripe cocoa seeds contain active proteases (Biehl & Passern, 1982). The rate of release from proteins of individual amino acids and peptides and the relative amino acid composition may vary during the course of fermentation due to the activity and the cooperation of endo- and exopeptidases. The activity of the proteases depends on progressive enzyme inhibition (Forsyth *et al.*, 1958) and on the variable decrease of the pH-value in the nibs when acetic acid is absorbed after seed death from the fermenting pulp (Biehl & Passern, 1982; Biehl *et al.*, 1982*b*, 1985; Meyer *et al.*, 1989).

Thus, the final amount and composition of free amino acids in fermented cocoa beans may depend on the kinetics of amino acid release under these variable conditions. Since proteolysis is almost completed within two days after seed death during normal fermentation (Forsyth *et al.*, 1958), in this paper the kinetics of proteolysis during a first (premortem) phase of 20 h and a subsequent (post-mortem) proteolytic phase of 48 h is followed in fermentation-like seed incubations by amino acid analyses using HPLC.

MATERIALS AND METHODS

Material

Ripe cocoa pods (mixed hybrids) from the normal harvest of the MARDI Cocoa and Coconut Research Division, Hilir Perak, Malaysia, were sent to Braunschweig. Material A was harvested in July 1987, Material B in August 1986. Seeds from Material A were extracted from the pods 10 days after harvest for incubation or lyophilization, respectively. Seeds from Material B were lyophilized within 3 to 5 days after harvest for acetone dry-powder preparation.

Incubations

Incubation of whole seeds

With Material A, aseptic incubations of whole seeds without pulp in buffer

solution in a nitrogen atmosphere under conditions equivalent to fermentation were performed as described previously (Biehl & Passern, 1982; Biehl *et al.*, 1985; Kirchhoff *et al.*, 1989). A first incubation at 40°C (premortem condition) was followed by a second incubation at 50°C (post-mortem, acidic condition) in media specified in the legend of Table 1.

For each sample the first incubation ran 20 h with 60 g of seeds in 90 ml of medium. Then the seeds were separated from the medium. The second incubation ran 0, 4, 12, 24 and 48 h, respectively, in 300 ml medium at each of the different stages A_1 to A_5 (Tables 1–3). The seeds and the media were then separated quantitatively, immediately frozen, stored at −20°C and were lyophilized to <3% water content.

Acetone dry-powder incubations

Forty-two grams of acetone dry-powder was prepared from 210 g of dried cotyledons from Material B. Cotyledons were separated from lyophilized unfermented seeds and were ground and weighed. Equivalent amounts were used for acetone dry-powder preparation. Extraction of fat with petroleum benzene and subsequent extraction with acetone and acetone/water-mixtures containing 0·1% of thioglycollic acid were performed as described previously (Kirchhoff *et al.*, 1989) but four instead of eight extractions with acetone/water-mixtures were carried out. Five milligrams of the acetone dry-powder were suspended in 1 ml McIlvaine-buffer (200 mM; pH 4·5 or 5·5 respectively). The suspensions were homogenized for 5 min in a Potter-Elvejham homogenizer and were subsequently incubated at 55°C for 4 h.

Analyses of free amino acids by HPLC

Extraction and purification of free amino acids

Seeds. The quantitatively combined acetone- and acetone/water-extracts resulting from the acetone dry-powder preparation of cotyledons were collected. Acetone was evaporated and polyphenols were removed using polyamide.

Media. The fraction of the freeze-dried media which is soluble in 70% v/v acetone/water was used. Polyphenols were separated with polyamide. These methods are described in detail elsewhere (Kirchhoff *et al.*, 1989).

Incubated acetone dry-powders. After incubation 1·0 ml of the homogenized suspension of powder plus medium was added to 4·0 ml ice-cold acetone. After standing for 30 min at 0°C the suspension was centrifuged at 6000*g* (15 min). Aliquots of 500 µl were dried under reduced pressure.

Determination of free amino acids (HPLC)

Conversion into *o*-phtalaldehyde (OPA) derivatives, reversed-phase HPLC,

TABLE 1
Changes in the Composition of Free Amino Acids during Fermentation-Like Incubation of Cocoa Seeds

	Stage					
	A_0	A_1	A_2	A_3	A_4	A_5
First incubation (h)	0	20	20	20	20	20
Second incubation (h)	0	0	4	12	24	48
Amino acid (mol %)						
Acidic	74·4	47·7	31·1	21·5	20·4	16·8
asp	21·6	1·24	1·83	1·61	1·38	1·79
glu	25·7	28·5	8·14	5·92	5·55	6·07
asn	22·2	12·2	14·2	10·2	10·4	6·78
gln + his*	4·92	5·72	6·91	3·71	3·13	2·15
Hydrophobic	18·7	43·4	54·6	64·1	64·1	63·6
leu	0·00	2·22	6·08	12·6	15·4	18·3
ala	7·58	21·2	24·1	20·7	18·8	14·1
phe	1·46	3·07	6·05	10·6	10·9	12·6
tyr	6·91	10·5	11·0	11·2	10·9	9·58
val	1·26	3·10	4·30	5·37	4·99	5·45
ile	1·44	3·33	3·13	3·53	3·14	3·67
Others	6·98	8·96	14·3	14·5	15·5	19·6
lys	0·00	0·00	0·00	0·95	2·08	2·68
arg	1·47	1·17	2·33	2·43	3·01	5·24
his*	/	/	/	/		
thr	0·52	1·54	2·27	2·45	2·05	2·23
ser	3·69	2·64	4·50	3·18	2·89	3·36
gly	1·30	2·11	3·34	2·76	2·02	2·36
met	0·00	0·19	0·89	1·66	1·93	2·27
trp	0·00	1·3	0·97	1·02	1·52	1·43
Total amino acids (μmol/g freshweight)	7·67	3·62	9·85	12·5	16·2	19·4

Whole seeds were incubated and amino acids were determined (HPLC) at five stages of incubation (A_1 to A_5). The data include the sum of amino acids in the seeds and in the incubation media (exudates). A_0 represents free amino acids in the cotyledons of unfermented seeds before incubation. The composition of amino acids at each stage is given in mol-% of estimated total amino acids (100%). The absolute amount of total amino acids is given in μmol/g wet weight of the seeds before incubation. Conditions: first incubation at 40°C for 20 h in citric acid/NaOH-buffer (35 mM; pH 5·5); second incubation at 50°C for up to 48 h (as indicated) in acetic acid/NaOH-buffer (200 mM; pH 4·5).

* glutamine and histidine were not separated by HPLC and are both included in the sum of acidic amino acids.

TABLE 2

Absolute Changes in Free Amino Acids during Each Interval of Fermentation-Like Incubation of Cocoa Seeds

	Interval					
	A_0	A_1-A_0	A_2-A_1	A_3-A_2	A_4-A_3	A_5-A_4
First incubation (h)	0	20	20	20	20	20
Second incubation (h)	0	0	4	12	24	48
Amino acid	*Original content*	*Increase (+) or decrease (−) per interval*				
Acidic	5·70	−3·98	+1·34	−0·39	+0·62	−0·04
asp	1·65	−1·61	+0·14	+0·02	+0·02	+0·13
glu	1·97	−0·94	−0·23	−0·06	+0·16	+0·28
asn	1·70	−1·26	+0·96	−0·13	+0·40	−0·36
gln/his*	0·38	−0·17	+0·47	−0·22	+0·04	−0·09
Hydrophobic	1·43	+0·14	+3·81	+2·61	+2·40	+1·99
leu	0·00	+0·08	+0·52	+0·97	+0·92	+1·06
ala	0·58	+0·18	+1·61	+0·21	+0·46	−0·31
phe	0·11	0·00	+0·48	+0·73	+0·45	+0·68
tyr	0·53	−0·15	+0·70	+0·32	+0·36	+0·10
val	0·10	+0·02	+0·31	+0·25	+0·14	+0·25
ile	0·11	+0·01	+0·19	+0·13	+0·07	+0·21
Others	0·54	−0·20	+1·09	+0·38	+0·71	+1·28
lys	0·00	0·00	0·00	+0·12	+0·22	+0·18
arg	0·11	−0·07	+0·19	+0·07	+0·18	+0·53
his*						
thr	0·04	+0·02	+0·17	+0·08	+0·03	+0·10
ser	0·28	−0·19	+0·35	−0·05	+0·07	+0·18
gly	0·10	−0·02	+0·25	+0·01	−0·02	+0·13
met	0·00	+0·01	+0·08	+0·12	+0·11	+0·13
trp	0·00	+0·05	+0·05	+0·03	+0·12	+0·03
Total amino acids	7·67	−4·05	+6·24	+2·60	+3·73	+3·23

Data are taken from the same experiment as in Table 1, but the changes in the amounts of amino acids (in cotyledons plus media) from one stage of incubation to the next (intervals $A_{i+1} - A_i$) is given in μmol per g wet weight of the untreated seeds. See footnote of Table 1 for further details.

TABLE 3
Increase and Decrease of Free Amino Acids during Four Hours of Acidic Proteolysis in Whole Seeds and in Acetone Dry-Powders (acp)

	Whole seeds		*Acetone dry-powder*			
	A_2-A_1		$B_{(4\cdot5)}$		$B_{(5\cdot5)}$	
pH (medium)	4·5		4·5		5·5	
Incubation temperature	50°C		55°C		55°C	
Amino acid	*μmol/g acp*	*mol %*	*μmol/g acp*	*mol %*	*μmol/g acp*	*mol %*
Acidic	9·84	21·5	0·50	3·6	0·10	0·5
asp	1·00	2·2	0·01	0·1	0·00	0·0
glu	−1·68	−3·7	0·06	0·4	0·10	0·5
asn	7·03	15·3	0·26	1·9	0·00	0·0
gln/his	3·49	7·6	0·17	1·2	0·00	0·0
Hydrophobic	28·0	61·1	9·35	69·4	18·4	89·5
leu	3·81	8·3	4·05	30·1	8·10	39·4
ala	11·8	25·8	0·51	3·8	1·90	9·2
phe	3·56	7·8	3·50	26·0	5·25	25·5
tyr	5·15	11·2	0·57	4·3	1·70	8·3
val	2·29	5·0	0·32	2·3	0·75	3·6
ile	1·38	3·0	0·40	2·9	0·70	3·4
Others	7·98	17·4	3·65	26·9	2·05	10·0
lys	0·00	0·0	2·19	16·3	1·15	5·6
arg	1·38	3·0	0·07	0·5	0·00	0·0
his*	/	/	/	/	/	/
thr	1·24	2·7	0·20	1·4	0·40	1·9
ser	2·56	5·6	0·19	1·4	0·00	0·0
gly	1·86	4·1	0·31	2·3	0·00	0·0
met	0·60	1·3	0·43	3·1	0·45	2·2
trp	0·35	0·8	0·26	1·9	0·05	0·2
Total	45·9	100	13·5	99·9	20·6	100

Changes in the amounts and in the relative proportions of free amino acids during 4 h of acidic incubations of whole seeds ($A_2 - A_1$) and of acetone dry-powders (acp) ($B_{(4\cdot5)}$; $B_{(5\cdot5)}$) are shown. Although Materials A and B were from different samples of fresh cocoa pods and the incubation temperatures were slightly different, this table allows us to compare proteolysis in whole seeds with that in acp and illustrates the effect of pH on amino acid liberation. In the acp, the actual pH-value was 4·5 ($B_{(4\cdot5)}$) or 5·5 ($B_{(5\cdot5)}$), respectively, while in the whole seeds the (average) pH was changing from 6·5 to 5·2 during the 4 h of incubation, the pH of the medium remaining constant at pH 4·5 (Fig. 3). The amino acids were determined in media + cotyledons (or acp respectively) after incubation. Data of the first column ($A_2 - A_1$) are from Table 2 but are calculated in μmol per g acp of the untreated material. The dry weight of acp of Material A was determined separately. For further details see Table 1.

fluorimetric detection and standard deviations are dealt with elsewhere (Kirchhoff *et al.*, 1989). For the determination of the low amounts of amino acids after acetone dry-powder incubation the procedure was modified: The dried 500 µl aliquot was mixed with 250 µl of a freshly prepared mixture of 1·0 ml OPA-reagent and 3·0 ml reaction buffer. The mixture was injected and allowed to react for 2 min, then the reaction was stopped by flushing the injection loop with the starting eluent and beginning the chromatographic separation. With this material (B), the standard deviations were four times higher than reported previously (Kirchhoff *et al.*, 1989) because of the low concentration of amino acids in the injection-solutions.

Determination of nitrogen

Total free α-amino nitrogen (with ninhydrin) and total peptide-bound nitrogen (Kjeldahl) were determined after precipitation of proteins with trichloroacetic acid (TCA) from solutions prepared during the course of seed incubations and during acetone dry-powder incubations.

Preparation of solutions

Incubations of seeds. The freeze-dried samples of seeds and of media after incubation were prepared separately.

Media. The aqueous solutions of the freeze-dried, polyphenol free media, prepared as described above were mixed (1:1 v/v) with aqueous TCA-solution (20%) and centrifuged.

Seeds. The acetone/water extract and the acetone dry-powders were prepared from cotyledons (see above). The acetone dry-powder was extracted twice with 80 ml and once with 40 ml of distilled water (adjusted to pH 5·5) per g acetone dry-powder. Proteins were precipitated from the combined extracts as described above. The acetone/water extracts, after removal of polyphenols and acetone, were applied to an ion-exchange column to remove thioglycollic acid, added during acetone extraction, which would interfere in the ninhydrin reaction.

A strong cation-exchange column was prepared (Amberlite IR-120, sodium form; 20 mm width, 92 mm height; equilibrated with 1N HCl to pH 1). Distilled water was passed through the column, raising the pH-value to 5·5. After application of the sample, the column was adjusted to a flow rate of 1 drop/s and was washed with water (100 ml), isopropanol/water (first 1:1 v/v; 50 ml, then 8:2 v/v; 50 ml) and again with distilled water (400 ml). The washings were discarded. Peptides and amino acids were eluted with aqueous ammonia (pH > 10) (3M, 40 ml) and subsequently with distilled water (50 ml). The eluates were combined quantitatively. Ammonia was removed under reduced pressure. The volume of the remainder was adjusted

with distilled water to the original volume of the sample before its application to the column. This solution was subjected to the TCA-treatment as described above.

Incubations of acetone dry-powders. One millilitre of the suspension of acetone dry-powders in the incubation medium was mixed with 100 μl of an aqueous TCA solution (50%). After one hour of storage at 0°C, the proteins were precipitated by centrifugation.

Determination of peptide- and α-amino nitrogen

Nitrogen was determined in the TCA-supernatants of extracts from (a) medium plus cotyledon acetone dry-powder plus acetone/water extracts after seed incubations and (b) from suspension of acetone dry-powder in media after acetone dry-powder incubations. 'Peptide nitrogen' stands for the difference between total nitrogen and purine nitrogen in the protein-free extracts. Total nitrogen was determined by the micro-Kjeldahl method (Lieb, 1931). Purines were determined separately by HPLC and purine nitrogen was calculated. 'Free α-amino nitrogen' is derived from the ninhydrin colour-reaction of the free amino acids and peptides in these extracts, calculated from a leucine standard. The method of Moore and Stein (1954) as modified by Quesnel (1970) was applied.

Determination of purines

The acetone/water extracts from seeds and the incubation media after removal of polyphenols (see above) contained theobromine and caffeine. For determination of these purines a portion of the respective extract was shaken with a threefold volume of $CHCl_3$ for 5 min. The organic phase was collected quantitatively. The procedure was repeated seven times and the organic phases pooled. $CHCl_3$ was removed under reduced pressure and the residue was solubilized in a volume of distilled water equivalent to that of the extract before purine extraction. The purines were separated and estimated by reversed-phase HPLC (detection at 280 nm). Column: Shandon Hypersil ODS; 5 μ-material; $l = 240$ mm; $\phi = 4{\cdot}6$ mm; precolumn: Shandon Hypersil ODS; 10 μ-material; $l = 20$ mm; $\phi = 4{\cdot}6$ mm; eluent A: 1 ml trifluoracetic acid in 1000 ml distilled water; eluent B: 1000 ml acetonitrile + 10 ml distilled water + 1 ml trifluoroacetic acid; flow rate: 1·2 ml/min. Gradient: in 20 min from 100% A to 60% . The standard deviation of the quantitative estimation was $\pm 2\%$.

Extraction and electrophoretic separation of cocoa seed proteins

Proteins in acetone dry-powders of cotyledons were solubilized at 95°C for 3 min in a phosphate buffer (12.5 mM NaH_2PO_4, 37 mM Na_2HPO_4; pH 7·2)

containing 10 mg/ml sodium dodecylsulfate (SDS) and 143 mM mercaptoethanol (ME) as described previously (Biehl *et al.*, 1982*a*). Ten microlitres of the clear supernatant after centrifugation, equivalent to 100 μg acetone dry-powder, was applied to a discontinuous SDS-PAGE, using the method of Laemmli (1970), which gives higher resolution than the method of Shapiro *et al.* (1967) which we used previously (Biehl *et al.*, 1982*a*, 1985). The gel concentrations were 5% for stacking and 15% for separation. Buffer concentrations were reduced to 50% of those given in the literature. Marker proteins were purchased from 'Pharmacia' and included phosphorylase *b* from rabbit muscle (94·0 kD), albumin from bovine serum (67·0 kD), albumin from egg white (43·0 kD), carbonic anhydrase from bovine erythrocytes (30·0 kD), trypsin inhibitor from soy bean (20·1 kD) and α-lactalbumin from bovine-milk (14·4 kD). Four micrograms of each protein were subjected to the gel. The proteins were stained with Coomassie-brilliant-blue.

Water content and pH-values in seeds were determined as described by Kirchhoff *et al.* (1989).

RESULTS

Proteolysis in intact seeds takes a different course from proteolysis in acetone dry-powder (acp), presumably as a result of structural peculiarities in the seed mesophyll-cells (Biehl, 1973; Biehl & Passern, 1982; Biehl *et al.*, 1982*a*,*b*). Proteolysis was studied in seeds and in acp.

Incubation of whole seeds

During normal fermentation, the seeds are alive during the first day. Subsequently they die, due to the increase in temperature and the up-take of acetic acid (Quesnel, 1965). The first incubation in citric acid/NaOH-buffer at 40°C simulates the premortem phase, whereas the second incubation in acetic acid buffer at 50°C represents the post-mortem proteolytic phase (Biehl & Passern, 1982). In contrast to previous investigations, in this study, the free amino acids in the seeds plus those exuded from the seeds into the fermentation medium were determined, since the process of proteolysis rather than the accumulation of amino acids in the seeds was what interested us.

As shown in Fig. 1(a) (see also Table 1, last line) the absolute amount of total free amino acids decreases strongly during the first incubation, (A_0 to A_1) but increases continually during the second incubation (A_1 to A_5). It is also evident from Fig. 1(a) that free acidic amino acids but not the non-acidic

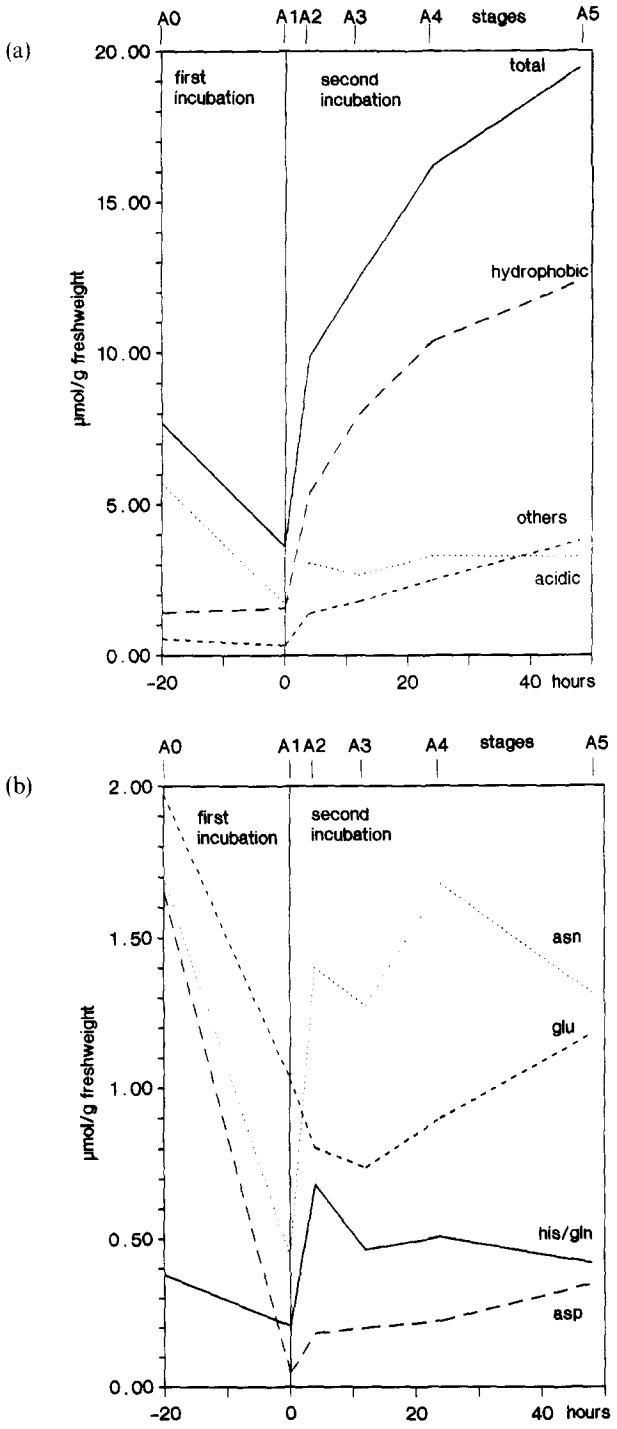
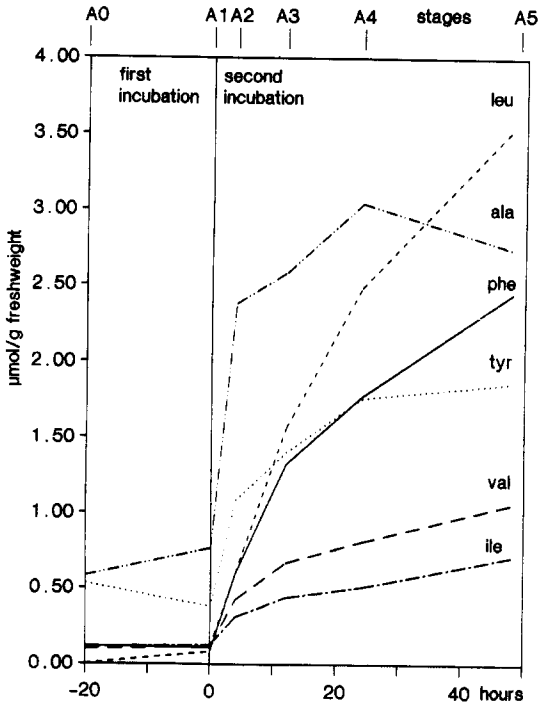


Fig. 1. Kinetics of amino acid turnover during fermentation-like seed incubation of whole cocoa seeds. The amount of free amino acids (μmol per g wet weight of the seeds before incubation) in cotyledons plus medium at each stage (A_0 to A_5) of incubation is plotted against time of incubation. (a) Total and groups of amino acids; (b) acidic. This incubation and further details are described in the footnote to Table 1.

(c)



(d)

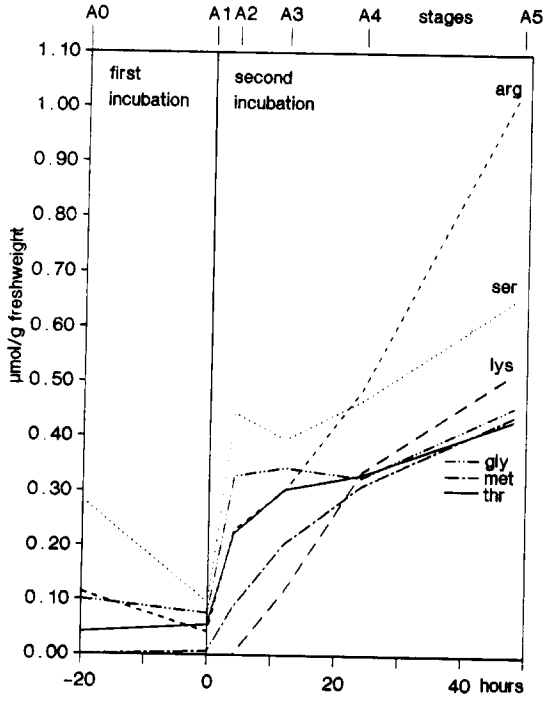


Fig. 1.—*contd.* (c) Hydrophobic; (d) other amino acids.

amino acids are reduced during the first (premortem) incubation, when no proteolysis takes place. The specific degradation of the free acidic amino acids of unfermented seeds before the start of proteolysis is one, but not the only reason (see below), for the low amount of acidic compared to hydrophobic amino acids in fermented cocoa (Kirchhoff *et al.*, 1989; see also Table 1, A_4, A_5). The rate of formation of total free amino acids during subsequent proteolysis peaks during the first four hours of the second incubation and steadily decreases thereafter. However, the rate is quite different for each group of amino acids, acidic, hydrophobic and others (Fig. 1(a)). About 60% of amino acids released during the 48 h of the second incubation are hydrophobic. Thus, in addition to the degradation of acidic amino acids during the premortem phase, the predominant release of hydrophobic amino acids during proteolysis is responsible for the high proportion of these amino acids after fermentation. Also, differences in the rate of formation of individual amino acids within the different groups (acidic, hydrophobic and others were found (Fig. 1(b)–1(d)). Thus, there is no constant proportion of amino acids released during acidic proteolysis in the seeds. By far the greatest increase in total free amino acids is due to the release of leucine, alanine, phenylalanine and tyrosine (Fig. 1(a), 1(c)). The composition of free amino acids (in mol-%) varies at each stage of the incubation being analyzed (Table 1). The slopes of the curves (Fig. 1(b)–1(d) and Table 2) reveal that the release of individual amino acids depends on the progress of proteolysis. Focusing on the hydrophobic amino acids (Fig. 1(c) and Table 2), it can be seen that during the first 4 h the rate of release of alanine is higher than that of leucine, but later on the situation is reversed.

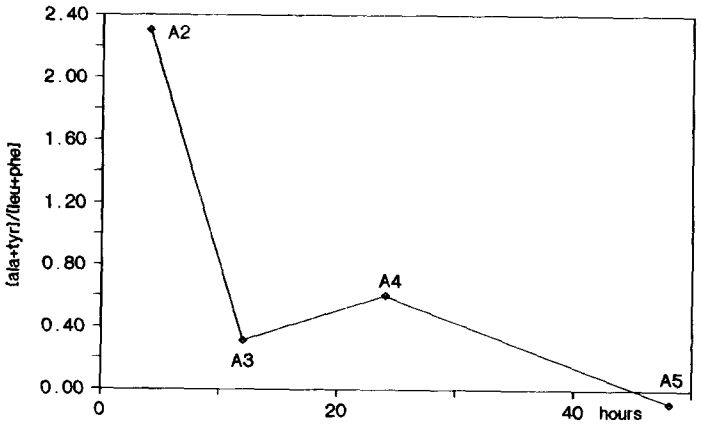


Fig. 2. The ratios are calculated from data in Fig. 1(c). The time of the second incubation is considered. For ala there was a decrease during the last incubation interval (A_5–A_4). The sum of (ala + tyr) was negative; thus the ratio (ala + tyr):(leu + phe) was negative.

This holds for other amino acids as well. Figure 2 shows the drastic change in the ratio of (alanine + tyrosine) to (leucine + phenylalanine) during proteolysis. The irregular progress of the changing amounts of acidic amino acids and their amides (Fig. 1(b)) in contrast to other amino acids indicates a more complex metabolism than just their progressive release from proteins (perhaps overlapping of degradation and formation).

Protein degradation during seed incubation was followed by SDS-gel electrophoresis in order to find out if all seed proteins are subjected to enzymatic digestion. The incubation media were not analyzed, since previous investigations had shown that they do not contain proteins after incubations (Biehl *et al.*, 1982*a*). Figure 3 gives a schematic representation of the reduction of protein peptides through the stages of incubation. During incubation in this strongly acidic medium (see legend Table 1) most of the peptides ≥ 20 kD are degraded (stage A_4). The vacuolar storage proteins, represented by Bands VII and IX (Biehl *et al.*, 1982*a*), are the first to be attacked (stages A_1 to A_2), with other protein peptides (e.g. Band X) following on.

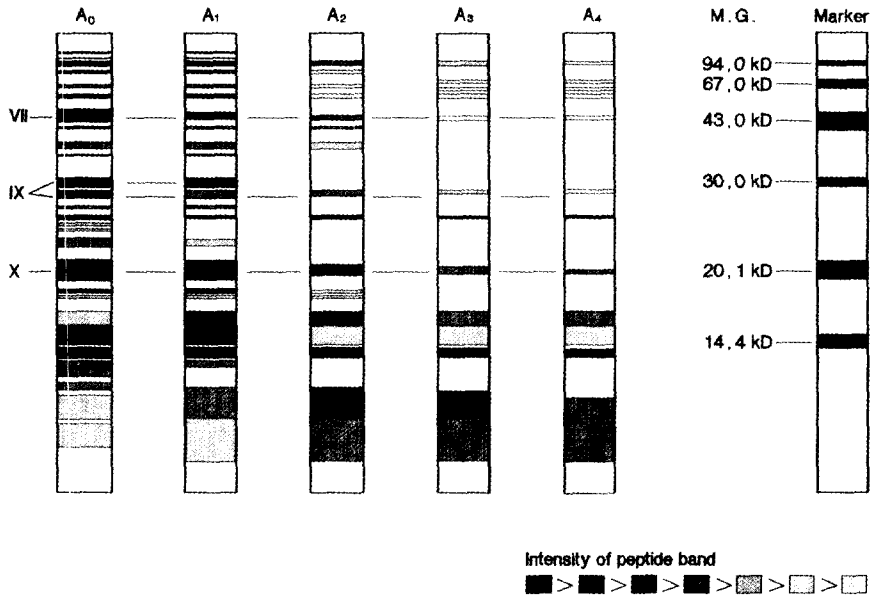


Fig. 3. Proteins of incubated seeds were extracted at each stage (A_0–A_5) of the experiment described in Table 1. About 85% of seed proteins are extracted with SDS-solution. The peptide pattern after SDS-gel electrophoresis of the protein extracts from stages A_0–A_4 are shown in a schematic representation. In each pherogramme the protein extract equivalent to 100 μg of acetone dry-powder was applied. Intensity was estimated visually. Bands VII and IX represent peptides of vacuolar storage proteins, whereas Band X represents a prominent seed protein, not classified as a vacuolar storage protein (Biehl *et al.*, 1982*a*). The degradation of VII and IX precedes that of X.

Incubation of acetone dry-powder from seeds

The conditions under which the enzymatic reactions take place in the cellular structure of whole seeds are complex. The average pH value in the nibs does not decrease below 5·2 during the first four hours of incubation (Fig. 4), but the actual pH varies across the seed cross-section, forming a pH-gradient with pH 4·5 at the surface and pH 6·5 in the centre of the cotyledon tissue. pH 4·5 is established, not before about 40 h of acidic incubation. This is readily explained by the slow penetration of acid from the incubation medium into the seeds (Biehl *et al.*, 1982*b*). While the nib pH is not homogeneous in whole seeds it is so during the incubation of acetone dry-powder suspensions where the total protein of the acetone powder is directly exposed to the pH of the medium. Besides the absence of fat and polyphenols, complete structural desintegration is the prominent difference between acetone dry-powder and whole seed incubations.

In Table 3, the increase of free amino acids during the first four hours of acidic proteolysis in whole seeds (A_1–A_2) and in acetone dry-powder of unfermented seeds ($B_{4.5}$) are compared. In both cases, the same pH-value (4·5) was maintained in the incubation medium. In contrast to whole seeds, in acetone dry-powder the proportions of leucine, phenylalanine and lysine accumulated are significantly higher; those of aspartic acid, alanine and tyrosine are lower. Although hydrophobic amino acids dominate in both cases, the relative composition varies in respect to most of the individual amino acids. The ratio (ala + tyr):(leu + phe) considered before (Fig. 2) is as

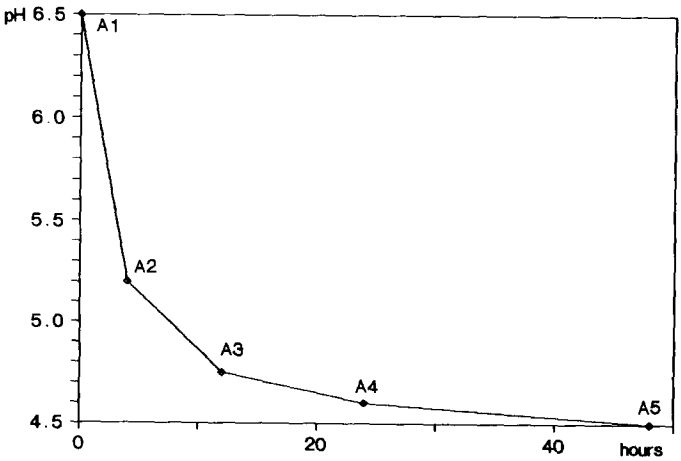


Fig. 4. In the incubation experiment described in the footnote of Table 1, the pH of the cotyledons was determined at each stage (A_1–A_5) of the second incubation in acetic acid/NaOH solution. 10 g of cotyledons were blended in 100 ml of water and the pH was determined in the homogenate.

low in acetone dry-powder after only four hours of incubation as in whole seeds after prolonged incubation (Fig. 1(b); Fig. 2; Table 2, A_5–A_4; Table 3, $B_{(4.5)}$).

Since these differences in the composition of free amino acids could be due to the higher actual pH value in the whole seeds during incubation, the effect of pH 5·5 compared to pH 4·5 during acetone dry powder incubation was studied (Table 3, $B_{(4.5)}$ and $B_{(5.5)}$). However, the data indicate that the increased pH-value causes changes in the relative amino acid composition which are quite different from those in whole seeds. It would appear, therefore, that a higher pH-value in the nibs was not responsible for the different composition of free amino acids released during incubation of whole seeds compared to acp.

TABLE 4

Determination of Nitrogen of the Peptides and Amino Acids released after Four Hours of Proteolysis in Whole Seeds and in Acetone Dry-Powder

		Material, incubated			
		Whole seeds ($A_2 - A_1$)		*Acetone dry-powder* (*acp*) ($B_{(4.5)}$)	
		mg/g acp	*% of total N*	*mg/g acp*	*% of total N*
I	Total N (Kjeldahl) in the acp prepared from the material before incubation	55·2	100	55·1	100
II	Release during incubation				
(a)	TCA-soluble N (Kjeldahl)	6·8	12·3	28·5	51·6
(b)	TCA-soluble α-amino-N (Ninhydrin)	1·9	3·4	4·6	8·3
(c)	α-amino-N of free amino acids (calculated from HPLC-analyses)	0·64	1·16	0·19	0·34

The incubation experiments are the same as in Table 3. To find the total amount of proteolytic products released during 4 h of incubation, the amount of α-amino-N in free amino acids (IIc, derived from HPLC-analyses) is compared to the amounts of Kjeldahl-N (IIa) and α-amino-N determined by the ninhydrin method (IIb) in the supernatants after precipitation of proteins with trichloroacetic acid (TCA). With incubated whole seeds, the difference between samples A_2 and A_1 are given, but the values are calculated per g acp prepared from the material before incubation. With incubated acetone dry-powder, the differences between the incubated and the nonincubated powders are given. All data are from cotyledons + media or acp + media, respectively. For details of incubation see footnotes of Tables 1 and 3. Far more peptides are released than free amino acids during proteolysis. In view of the low relative amounts of basic amino acids (Table 1) the difference of IIa minus IIc (Table 4) gives roughly the amount of peptide-bound nitrogen.

The much lower amount of total amino acids liberated in acp than in whole seeds (Table 3, last line) implies enzyme inactivation during preparation of acetone dry-powder from cocoa seeds. Nitrogen determinations (Table 4) show that although the amount of amino acids released is reduced (Table 4, IIc) the total proteolysis (release of amino acids plus peptides, Table 4, IIa,b) is much higher in acp compared to whole seeds. It seems that exopeptidases releasing amino acids, but not endopeptidases releasing peptides, are inhibited in part during preparation of acp, and that the cooperation of all proteases present in the fresh seed is responsible for the composition of the free amino acids.

DISCUSSION

Both flavour potential and proteolysis in cocoa seeds have been shown to depend on variable, interrelated processes during fermentation, such as nib acidification, premortem germination-like reactions or changes in the subcellular structures (Biehl *et al.*, 1985). These processes are assumed to affect the rate of release of free amino acids and peptides (De Witt, 1957; Biehl, 1961; Seiki, 1973; Biehl & Passern, 1982) and the composition of free amino acids.

Some details of the effects of these processes on proteolytic amino acid release are shown in this paper:

1. The specific reduction of acidic amino acids takes place in the living seed during a premortem phase before post-mortem proteolysis. The seeds are alive under the conditions of the first incubation (Biehl *et al.*, 1977). Seiki (1973) found a decrease of free glutamic acid during the first day of heap and tray fermentation, when generally the seeds are still alive. It seems, however, that the reduction of acidic amino acids is not bound to the living seeds, since we found degradation of acidic amino acids also during the same type of (non acidic) preincubation at 50°C (data not shown), at which temperature the seeds are rapidly killed (Biehl *et al.*, 1977).
2. The dominance of leucine, phenylalanine, alanine and tyrosine under the free amino acids in fermented cocoa (Kirchhoff *et al.*, 1989) is due to both the premortem degradation of acidic amino acids and to the specific release of hydrophobic amino acids during subsequent proteolysis. This is supported by our recent finding (Biehl *et al.*, 1989), that all endopeptidase activity in ripe cocoa seeds is due to aspartic acid proteinase, which splits peptide chains between hydrophobic amino acids (Belitz & Grosch, 1982).

3. In whole seeds, the rate of amino acid release during post-mortem proteolysis is not linear during the 20 or 40 h of incubation, but peaks within 5 h and thereafter progressively declines. Exhaustion of suitable substrates for specific exopeptidases is one possible explanation. Additionally, studies of Forsyth *et al.* (1958) on protease inactivation during fermentation, as well as the sensitivity of exopeptidases to acetone-treatment, support the assumption that enzyme inactivation is responsible.
4. The composition of free amino acids released during proteolysis changes continually during the 48 h of seed incubation (Fig. 1, Fig. 2). Thus the final amino acid composition depends on the time the process was allowed to continue. It depends, also, on the nib pH-value (Table 3). In whole seeds, the nib pH changes continually during proteolysis in acid media (Fig. 4). However, some peculiarities remain unexplained. The ratio (ala + tyr):(leu + phe) is high during the initial stages of proteolysis in whole seeds but low later on (Tables 1 and 2) and seems not to depend on pH-differences (Table 3). One explanation is that the substrate protein available changes during the second incubation. The changing pattern of protein peptides (Fig. 3) during incubation supports this assumption.

The storage proteins (VII, IX in Fig. 3) were degraded mainly in the initial stage (A_1–A_2), while digestion of other seed proteins (e.g. Band X, Fig. 3) was more pronounced later on. In two earlier publications we have shown that in low concentrations of acetic acid, the distribution of fused lipid bodies on the subcellular level restricts substrate accessibility to the proteases. Predominantly the storage proteins (VII, IX) are degraded. However, high concentrations of acetic acid cause a bulk separation of proteins and lipids and make all cell proteins accessible to proteolytic attack (Biehl *et al.*, 1982*a*,*b*). In the experiment described here, a high concentration of acetic acid was used in the incubation medium, which may be responsible for the degradation of nearly all seed proteins during the final stages of incubation.

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