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Changes in Cocoa Proteins during Ripening of Fruit, Fermentation, and Further Processing of Cocoa Beans

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During ripening of cocoa fruit the protein content of seeds decreased 25%, but consistent qualitative trends were not apparent. Most of the protein of unfermented beans could be solubilized and recovered. However, at the conclusion of fermentation only about one-third of the protein could be extracted from beans. The extractable protein fractions became progressively less pure during fermentation and at the end were only 40% protein. Fewer, more diffuse protein bands were evident on disc gels as fermentation advanced. Most of these changes are believed to involve protein interaction with polyphenols. Somewhat similar trends were noted during roasting and conching.

A recent study (Zak and Keeney, 1976) revealed protein differences between the two fundamental types of cacao, white Criollo and purple, pigmented Forastero. Compared to the latter, less than half as much protein could be solubilized and extracted from Criollo cocoa beans and, after a series of steps to remove contaminants from the extract, the protein content of the final dehydrated material was only about one-half that obtained from Forastero varieties. Moreover, electrophoresis of Criollo material yielded fewer protein bands and distinct differences were found when classified according to solubility characteristics. These differences relate to both genetically derived protein variations and the effects of post harvest events, especially phenolic tanning reactions.

This presentation is a complement to the above study. Using almost identical procedures, information was collected concerning protein changes during ripening of cocoa fruit, post harvest fermentation of cocoa beans, and key processing steps in the chocolate factory, namely, roasting and conching.

Cocoa fruit (pods) require 4–5 months to grow to full size following pollination (Seeschaaf, 1971). At this time the beans contained therein have also reached near maximum development. The pods are then left on trees to ripen for about a month before being harvested. During ripening the mucilaginous pulp surrounding the beans undergoes changes critical to a successful fermentation, primarily an increase in fermentable carbohydrate (Rohan, 1963).

After harvesting, the seeds and mucilaginous pulp from opened pods are fermented several days before the beans are dehydrated. Fermentation results in the formation of precursors of essential aroma compounds eventually generated by roasting in the chocolate factory. Fermentation also brings about a suppression of astringency and bitterness. Proteolysis, enzymatic and nonenzymatic browning, and tanning are important sequences in the development of chocolate flavor and other characterizing properties identified with chocolate.

Birch (1941) reported a significant loss of protein nitrogen during fermentation, only a portion of which would be accountable to proteolysis or diffusion through the testa. As suggested by DeWitt (1957), a more imporant contributor would be protein insolubilization caused by oxidation and tanning reactions involving polyphenols and polyphenol oxidase. Forsyth et al. (1958) showed that protein–polyphenol interactions do occur during fermentation to reduce protein solubility.

Polyphenols combine with proteins in a manner analogous to tannins converting animal hides to leather (Forsyth and Quesnel, 1963). This involves reversible complexing through hydrogen bonds, and irreversible oxidation of polyphenols to quinones followed by covalent condensation of quinones with reactive groups of amino acids, peptides, and proteins (Loomis, 1969). The latter most probably is the cause of the limited solubility of protein in fermented cocoa beans. A detrimental result of tanning is the poor biological value reported for cocoa beans (Lanteaume et al., 1972).

Tanning does, however, have effects on chocolate which are advantageous. The burnt feather taste of roasted protein is depressed as is the astringency associated with polyphenols (Forsyth and Quesnel, 1963). Thus, from the standpoint of flavor these interactions are desirable to a certain extent.

The processing of cocoa beans in the chocolate factory involves roasting followed by grinding of the cotyledon or nib portion to yield chocolate liquor, which may be separated into cocoa butter and cocoa fractions. Chocolate

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is obtained by mixing, refining, and conching a blend of chocolate liquor, cocoa butter, and sugar, with or without milk solids.

The importance of roasting has been referred to previously. Conching is a process in which molten chocolate is under continuous movement or agitation for a period ranging from a few hours to several days, during which unwanted aromas are volatilized, and oxidation along with other poorly understood chemical reactions occur. The net effect of conching is a mellowing of the flavor of chocolate. Aeration and maximum surface exposure appear to be especially important.

MATERIALS AND METHODS

Source of Samples. Samples for a study of protein changes during ripening of cocoa fruit were collected at the Cocoa Research Center, Itabuna, Bahia, Brazil. The pods were opened and testa and adhering pulp material were peeled from the cotyledons, which were then dried at 60 °C under reduced pressure.

Cocoa beans used in the fermentation study were supplied by V. C. Quesnel, Cocoa Research Department, University of the West Indies, Trinidad. Samples were taken daily during a 6-day, sweat box fermentation.

A comparison between unroasted and roasted cocoa beans was made using Bahia beans roasted 30 min at 150 °C in a laboratory, forced air oven. Conching was evaluated using commercial sweet chocolate conched 24 h at a paste temperature of 65 °C in a Petsholtz PVS 100 vertical conche.

Protein Recovery and Analysis. Procedures reported by Zak and Keeney (1976) were followed to recover protein fractions from cocoa beans and sweet chocolate. This included extraction of fat with ethyl ether and polyphenols with methanol. Defatted cocoa mass (0.5–1.0 g) with most polyphenolic material removed was extracted with acetic acid–urea–detergent solution, and the extract was filtered and concentrated after adsorption of residual phenols by a nonsoluble form of polyvinylpyrrolidone. A column of Sephadex LH20 was used to separate protein from nonprotein components, and the protein fraction was dialyzed and freeze dehydrated to yield a protein "prep".

Quantitative protein data were obtained for fractions off the Sephadex column by the method of Lowry et al. (1951) and by amino acid analyses for protein preps. Proteins in protein preps were separated by disc gel electrophoresis, and the preps were separated into solubility classes by successive treatment of undissolved material with water, 10% NaCl, 70% aqueous ethanol, and finally 0.2% NaOH. Again, methods followed were those described by Zak and Keeney (1976).

RESULTS AND DISCUSSION

Ripening. Results of protein analyses on cocoa beans from pods sampled throughout ripening are illustrated in Table I. Protein content of seeds decreased progressively between 135 and 160 days. Likewise, the amount of protein that could be solubilized decreased. Since the data were collected on a pooled weight basis rather than for individual seeds, it could not be determined if protein was being consumed during ripening or if other components in moisture-free defatted beans were increasing. The latter seems more likely. Schmieder (1976) found a slight increase in starch content during ripening in the same series of bean samples.

During ripening the purity of the protein prep material increased between 135 and 150 days and then decreased sharply in the later stages. Reasons for this are not clearly evident. Interpretation is complicated by the questionable validity of the value obtained for the 135-day sample, since it may have been an artifact related to the recovery of seed material from immature fruit. Whereas seeds from older fruit were obtained intact, those from the 135-day sample were fragile and shattered into spongy sections during removal of the testa. Disruption of cellular integrity and mixing of protein and polyphenols to form insoluble complexes is suggested. This could account for the low purity of the 135-day sample. Unfortunately, logistical problems vis-a-vis our laboratory and Brazil forced postponement of the confirmatory studies.

Table I. Protein Changes in Seeds of Cocoa Fruit during Ripening

	Days after pollination			
	135	143	150	160
Defatted cocoa bean mass				
Total protein,[a] %	23.6	21.6	21.4	17.6
Extractable protein,[b] %	14.2	13.2	12.5	11.5
Protein prep material				
Purity,[c] % protein	57.7	76.0	81.5	54.7
Solubility classification				
Albumin, %	42.4	57.0	57.0	44.2
Globulin, %	7.8	9.3	9.3	8.8
Prolamine, %	16.5	15.7	19.4	18.6
Glutelin, %	32.2	16.6	13.0	28.5

[a] From amino acid analysis of hydrolyzed defatted cocoa bean mass. [b] Protein off Sephadex by the method of Lowry et al. (1951). [c] From amino acid analysis of hydrolyzed prep material.

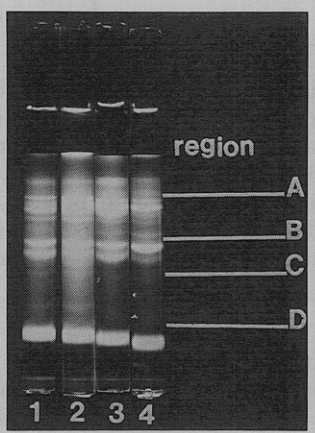

Figure 1. Disc gel electrophoresis of protein preps recovered from cocoa beans at different stages of ripening. Post pollination: (1) 160 days; (2) 150 days; (3) 143 days; (4) 135 days.

Solubility trends for protein preps during ripening show albumins and glutelins varying inversely to each other (Table I). The least and most ripe samples, 135 and 160 days, could be paired together according to similarity in solubility characteristics, as could the 143- and 150-day protein preps. Disc gels (Figure 1) do not show contrasting differences, although bands for preps of 143- and 150-day samples appear more diffuse and more complex than is the case for the 135-day and fully ripe, 160-day cocoa samples. This may reflect a concentration effect. The same amount of material was applied to all gels, but since samples of intermediate ripeness were more pure (Table I) a greater amount of protein was being applied to the gels for separation. Acid hydrolysis of protein preps followed by amino acid analysis did not reveal differences which could be related to stage of ripening. Amino acid patterns were similar to those reported by Zak and Keeney (1976).

Fermentation. Changes relating to protein parameters recorded for a series of samples collected during a fer-

Table II. Protein Changes in Cocoa Beans during Fermentation

	Fermentation days						
	0	1	2	3	4	5	6
Defatted cocoa bean mass							
Total protein,[a] %	17.6	17.2	20.4	20.7	21.2	20.8	20.1
Extractable protein,[b] %	17.1	15.4	12.8	10.4	10.0	8.0	7.4
Protein prep material							
Purity,[c] % protein	72.7	70.0	63.8	49.1	50.1	35.0	41.1
Solubility classification							
Albumin, %	41.3	47.3	56.1	72.5	68.5	69.2	70.7
Globulin, %	21.0	28.2	24.2	13.7	14.6	14.6	15.0
Prolamine, %	12.8	4.6	3.8	3.1	4.6	5.4	3.8
Glutelin, %	24.8	19.8	15.9	10.7	12.3	10.8	10.5

[a] From amino acid analysis of hydrolyzed cocoa bean mass. [b] Protein off Sephadex by method of Lowry et al. (1951). [c] From amino acid analysis of hydrolyzed prep material.

mentation of Trinidad cocoa beans are presented in Table II. The finding that total protein content changed only slightly during fermentation is in agreement with Maravalhas (1972). Except for a slight loss in the sweatings during the later stages of fermentation, degradation products are retained within the bean complex. The small increase in protein occurring early in the fermentation probably reflects microbial synthesis of amino acids and proteins in the pulp. The percentage composition of the amino acid fraction did not change appreciably during fermentation.

The large and almost linear decrease in extractable protein during fermentation (Table II) is contrary to the results of Niepage (1961) who reported increases in extractable protein during fermentation. It is believed his data are in error because the procedures employed would favor tanning and contamination of the extract. Our results conform with the sequences suggested by DeWitt (1957). Heat and acid generated by microorganisms cause bean death, after which polyphenols and proteins are released from their respective cells and tanning occurs. As tanning progresses, proteins become increasingly more insoluble. During the early stages, proteolytic enzymes hydrolyze proteins to more water-soluble components, smaller peptides, and amino acids (DeWitt, 1957; Roelofsen, 1958; Rohan and Stewart, 1967). This would account for a portion of the decrease observed, since these smaller components would not become part of the extractable protein fraction (Birch, 1941; Wolf, 1958).

The reduction in protein prep purity indicates progressively more contamination of the proteins with polyphenols. The contention of Birch (1941) that 50% of the protein is hydrolyzed or made an insoluble complex appears conservative. The first 1–3 days of fermentation are especially critical and coincide with bean death. Location in the fermentation heap influences when individual beans die (Lopez, 1974); thus, mass viability is quite heterogeneous in the early stages.

Although cocoa protein contains a full complement of amino acids, aspartic and glutamic acids together account for 25% of the total in fresh seeds (Zak and Keeney, 1976). Pronounced changes in these acids during fermentation were noted. Aspartic acid decreased progressively from 12.4 to 7.9% of total amino acids between 0 and 6 days. During this period glutamic acid increased from 15.4 to 26.2%. Generally, most other amino acids decreased, but less so than aspartic acid.

The reason for the increase in glutamic acid while most other amino acids were decreasing is not clearly understood. One possibility is that cocoa protein contains a substantial quantity of glutamine. Since amides of dicarboxylic acids show less reactivity with polyphenols (Mason and Peterson, 1965), glutamate accumulation might be expected during fermentation.

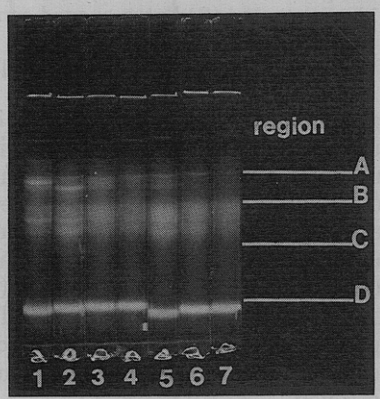

Figure 2. Disc gel electrophoresis of protein preps from cocoa beans at various stages of fermentation. Fermentation time left to right (1) through (7): 0, 1, 2, 3, 4, 5, and 6 days, respectively.

Table III. Protein Changes Caused by Roasting Cocoa Beans

	Raw	Roasted
Defatted cocoa bean mass		
Total protein,[a] %	18.4	17.1
Extractable protein,[b] %	4.2	3.5
Protein prep material		
Solubility classification		
Albumin, %	62.9	79.5
Globulin, %	12.1	3.6
Prolamine, %	14.2	6.8
Glutelin, %	10.9	9.7

[a] From amino acid analysis of hydrolyzed cocoa bean mass. [b] Protein off Sephadex by method of Lowry et al. (1951).

Proportioning the proteins of the prep material into solubility classes showed increases in the albumin fraction with accompanying decreases in globulins, prolamines, and glutelins in the early stages of fermentation. Albumin increases could reflect hydrolysis of water-insoluble proteins by proteolytic enzymes. Relative proportions did not change greatly after about the third day which could coincide with bean death and reduced proteolytic activity.

Disc gel electrophoresis of protein preps revealed subtle changes in proteins during fermentation (Figure 2). Bands from unfermented cocoa above region A and between A and B eventually disappeared as fermentation progressed. The most striking change occurred near 2–3 days when beans die. Below region B bands diffused and became fuzzy after 1–2 days. Similar results, noted for the proteins of heated milk, have been credited to interactions of protein components (Manning, 1969). For cocoa this may

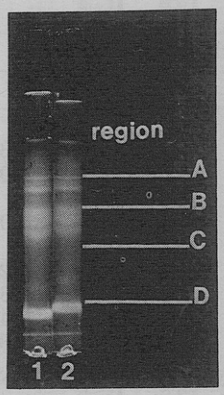

Figure 3. Disc gel electrophoresis of protein preps from unroasted (1) and roasted (2) Bahia cocoa beans.

reflect interaction of proteins and/or polyphenol complexing.

Roasting and Conching. As revealed in Table III roasting caused a slight decrease in the protein content of cocoa beans. In this study total protein was calculated from amino acid data for hydrolyzed samples and would include the contribution of free amino acids in beans. Involvement of free amino acids in nonenzymatic browning reactions during roasting is well documented (Bailey et al., 1962; Pinto and Chichester, 1966) and could account for most of the recorded decrease in protein content. Undoubtedly some protein amino acids, particularly lysine, are also destroyed.

Reduction of extractable protein during roasting is attributed to Maillard browning and further polyphenolic tanning. While enzymatic activity would not be expected, air roasting would provide ample oxygen for autocatalytic oxidation of polyphenols and continued tanning of proteins. Solubility studies of protein preps yielded trends similar to those observed during fermentation. The albumin content of the protein fraction of the protein prep increased from 63 to 80% balanced by decreases in globulins, prolamines, and glutelins. Disc gels (Figure 3) of Bahia beans showed the same protein bands, but they were less intense than in raw cocoa.

The total protein content of sweet chocolate was not changed appreciably by conching (Table IV). Values obtained through amino acid analyses, 4.3 to 4.7% of defatted sweet chocolate mass, are similar to the protein content estimated by assuming 28% liquor in the paste (information from supplier). If liquor is 10% protein (Watt and Merrill, 1963), defatted sweet chocolate would be 4.4% protein by calculation.

As indicated previously, the amount of extractable protein is reduced greatly by fermentation and roasting. Table IV shows that extractable protein values, already low due to these processes, are further reduced by conching. After 24 h of conching less than 10% of the protein in chocolate was recovered in the extractable protein fraction. A similar effect was noted for the purity of the protein prep. Only 5% of the prep material was protein after 24 h. Almost all of this protein fell into the water-soluble, albumin class, which again follows trends occurring during fermentation and roasting. Disc gels showed no distinct bands, only areas of limited fluorescence.

Table IV. Protein Changes Caused by Conching of Sweet Chocolate

	Conching time, h		
	0	15	24
Defatted sweet chocolate mass			
Total protein,[a] %	4.4	4.7	4.3
Extractable protein,[b] %	0.7	0.5	0.4
Protein prep material			
Purity,[c] % protein	10.9	9.8	5.0
Solubility classification			
Albumin, %	98.2	98.4	100.0
Globulin, %	1.8	1.6	0.0
Prolamine, %	0.0	0.0	0.0
Glutelin, %	0.0	0.0	0.0

[a] From amino acid analysis of hydrolyzed, defatted chocolate. [b] Protein off Sephadex by method of Lowry et al. (1951). [c] From amino acid analysis of hydrolyzed prep material.

SUMMARY

As shown through the data reported herein, cocoa protein undergoes progressive alteration during fermentation and roasting of cocoa beans, and when sweet chocolate is subjected to conching as a final process in its manufacture. Involved at various stages are partial hydrolysis of protein and sugar components, Maillard browning, and oxidative, phenolic tanning reactions. At the conclusion of processing essentially all of the protein exists in an insoluble, complexed form.

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