Extraction and Fractionation of Cocoa Proteins as Applied to Several Varieties of Cocoa Beans

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Protein was extracted from cocoa beans with a buffer solution and separated on Sephadex LH20. After dialysis and freeze dehydration protein preparations were characterized according to solubility in different aqueous media and electrophoretic behavior on disc gels. Compared to pigmented varieties, extracts from nonpigmented beans yielded only half as much protein off the Sephadex column, and nondialyzable solids were less pure with respect to protein (60% vs. 30% protein). Electrophoresis of pigmented varieties gave eight protein bands which were more intense and numerous than for nonpigmented types. Differences reflect protein–polyphenol interactions important to quality characteristics of chocolate, and methods used are suggested as adjuncts in breeding programs related to optimization of desirable quality characteristics.

Chocolate is manufactured from seeds of the species *Theobroma cacao*. From an evolutionary standpoint cocoa is derived primarily from two types of beans, Criollo and Forastero.

Criollo is characterized by pods which are yellow, rough surfaced, and somewhat pointed at the ends. Beans of freshly opened pods are nonpigmented and need only a relatively short fermentation period and mild roasting conditions to develop chocolate flavor. Historically, the flavor of Criollo beans has been recognized as especially desirable. Criollo's native habitat is west and north of the Amazon to Mexico.

Forastero, indigenous to the Amazon and Orinoco River basins, yields purple beans from pods which are smoother and more rounded than Criollo. Optimum flavor development through roasting necessitates a longer fermentation period than is the case for Criollo beans.

During the past century cacao has been transplanted to other warm, humid regions of the world, most notably West Central Africa. Natural adaptation and breeding programs directed toward disease resistance and increased production now yield genetically heterogeneous commercial crops.

Cocoa protein, 10% of dry bean weight, is influential in establishing quality characteristics in chocolate. Thermally initiated reactions of protein and its degradation products account for a significant number of the more than 300 compounds identified in the aroma fraction of chocolate (Keeney, 1972). Moreover, astringency associated with polyphenols is suppressed through complexes with protein and, under proper conditions of processing, undesirable flavor notes of protein are avoided (DeWitt, 1957; Purr et al., 1960; Bracco et al., 1969). Properties ultimately achieved represent the integrated effects of happenings in tropical regions where cacao grows and the subsequent processing in the chocolate factory.

Key events in cocoa bean exporting countries include ripening of fruit, harvesting, fermentation, and drying of cocoa beans. Traditional habits, growing conditions, and the variety of cocoa beans being harvested result in a lack of standardization in processing regimes among regions of production. Based on known characteristics, the chocolate manufacturer selects and blends beans, and then adjusts roasting conditions and the processes which follow to obtain the desired results.

The effects of various processes on protein hydrolysis and the accumulation of free amino acids have been followed through protein nitrogen determinations and amino acid analyses (Bracco et al., 1969; Maravalhas, 1972; Reineccius et al., 1972). However, there is a paucity of information about cocoa protein per se, nonhydrolytic changes, and other occurrences involving intact protein which might affect quality. Protein fractions have not been recovered in a state of purity sufficient for meaningful study.

The research reported herein was undertaken to develop suitable methods for extraction and purification of cocoa proteins. With this accomplished, varietal differences were studied. Another presentation covers processing parameters.

MATERIALS AND METHODS

Source of Samples. Cocoa beans of authenticated genetic origin were obtained from fruit selected by J. Soria, Interamerican Institute for Agricultural Sciences, Turrialba, Costa Rica. Upon arrival via air freight, the beans were removed from pods, immediately frozen, and then freeze dehydrated.

Pigmented (purple) beans included Nacional, UF 667, and Pound 7 as classified by Dr. Soria. Uf 667 and Pound 7 are of Forstero origin; Nacional may have both Forastero and Criollo factors. Criollo is the classical nonpigmented type while Porcelana and Catongo are Criollo–Forastero mutants.

Extraction of Proteins. A modification of the method of Meredith and Wren (1966) was developed to recover protein from cocoa beans after defatting by Soxhlet extraction with ethyl ether for 12–16 h.

Initially, polyphenolic material was extracted from 0.5–1.0 g of finely pulverized, defatted cocoa mass using redistilled methanol. The sample and 10 ml of solvent in a 15-ml centrifuge tube were sonicated 10 min in a 400-ml capacity sonic cleaning device filled with ice water, after which protein was sedimented using a clinical centrifuge. The supernatant solution containing polyphenols was decanted and discarded. This extraction process was repeated four times.

Protein was extracted from the sediment with 10 ml of buffer by sonication for 10 min. The buffer, pH 4–5, was

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0.1 M acetic acid, 3.5 M urea, 0.01 M hexadecyltrimethylammonium bromide, 0.1 M ascorbic acid, and 2% sodium ethylenediaminetetraacetate (EDTA). After centrifugation the supernatant protein solution was decanted. Extraction of the sediment was repeated 10 times to assure maximum recovery of protein.

The pooled protein extract was placed in an ice bath and 10 g of PolyClar-AT beads (GAF Corp.) was added to aid in the removal of additional polyphenolic substances. The beads had been cleaned prior to use by boiling in 10% HCl followed by washing with distilled water until chloride free. The protein extract, 90–95 ml, was filtered through Whatman 42 paper and the filter paper and beads were washed extensively with distilled water. The filtrate was then concentrated to 50–70 ml under reduced pressure in a rotary evaporator. Usually, the filtrate was fractionated on Sephadex within 2 h after extraction; when overnight storage was necessary it was refrigerated.

Separation on Sephadex LH20. A portion of the concentrated protein extract, 10–20 ml, was applied to a 5 × 50 cm Sephadex LH20 (Pharmacia Fine Chemicals, Inc.) column and eluted with a solution of 0.1 M acetic acid and 3.5 M urea. Flow from the column passed through a uv monitor (280 nm) coupled with a strip chart recorder and into a fraction collector set to take 10-ml fractions. Protein concentration in appropriate tubes was determined by the method of Lowry et al. (1951) using papain for the standard curve. All protein values determined by Lowry's method are expressed in papain equivalents.

The contents of the protein-containing tubes from the fraction collector, less the 1 ml used for protein analysis, were pooled, dialyzed against distilled water for 48 h, and freeze dehydrated. Hereafter, this dried cocoa protein material is referred to as "protein prep".

Protein Classification. Protein preps were classified according to solubility characteristics in the following manner. Protein prep, 5 mg, was hydrated with 5 ml of distilled water and filtered through Whatman 42 paper. A 2-ml water rinse of the sample container was also filtered, and then the filter paper was washed with 2 ml and 1 ml of H_2O. The total filtrate from the above treatment was called albumin or water-soluble protein. Material retained on the filter paper was washed with 5, 2, 2, and 1 ml of 10% NaCl to obtain a globulin fraction as a filtrate. Similar treatment with 70% aqueous ethanol followed by 0.2% NaOH yielded prolamine and glutelin fractions, respectively. The filter paper was always rinsed from the top to minimize chromatographic effects. Protein content in each fraction was determined according to the Lowry method. Based on summed protein values, recoveries of 92–105% were realized.

Amino Acid Analysis. Defatted cocoa mass, 40–50 mg, and protein preps, 4–5 mg, were hydrolyzed with 5 ml of 6 N HCl at 110 °C for 24 h in 10-ml sealed, evacuated ampules (Corning no. 12012). Norleucine internal standard had been added prior to sealing (5.0 and 1.5 μmol to bean and prep samples, respectively). Following hydrolysis, the contents were filtered and taken to dryness under reduced pressure to remove HCl. Water, 10–15 ml, was added and the samples again dried. Elapsed time between hydrolysis and analysis never exceeded 3 days, and storage was at 4 °C.

A Beckman Model 120C Autoanalyzer was employed to separate amino acids, using PS35 and UR30 resins for the basic and acidic plus neutral amino acids, respectively. Solutions suitable for injection were prepared by dissolving dried hydrolysates of cocoa mass and protein prep in 50 ml and 15 ml of water, respectively. Sample charge to the analyzer was 1 ml of solution.

Quantitation was accomplished with the aid of a computer and the internal standard. A standard curve for each amino acid was developed using 0.1 μmol of norleucine in combination with 0.01–0.20 μmol of amino acid. All amino acids showed linear relationships when peak area relative to norleucine was plotted vs. concentration. The slope of the curve for each amino acid and its molecular weight, along with the amount of norleucine added, were incorporated in a computer program to obtain molar and mass data.

Disc Gel Electrophoresis. Separation of cocoa proteins on 21% polyacrylamide gels was carried out according to a procedure for milk proteins described recently by Zak and Keeney (1974). The novel feature of this method is the use of Fluram (Hoffman LaRoche, Inc.) to produce fluorophores of proteins prior to electrophoresis. After separation, proteins appear as fluorescent bands under uv light without removing the gel from its tube and going through a staining–destaining process.

Molecular Weight Estimation. Molecular weight data for protein prep material were obtained by calibrating with standard proteins a 2.5 × 38 cm column of Sephadex G200, previously swelled in buffer 6 h on a boiling water bath. This buffer solution, 0.1 M acetic acid and 3.5 M urea, was the same as that used for eluting protein from the column. Absorption due to protein was monitored continuously at 280 nm as the eluent flowed to a fraction collector (3 ml/tube). Using elution volumes and log molecular weights as coordinates, ten standard proteins plotted essentially as a straight line. Two other proteins, cytochrome *c* and bovine serum albumin, had elution volumes corresponding to approximately twice the accepted molecular weights of 12000 and 69000, respectively. They may have been dimers. Andrews et al. (1964) suggested the precision of such molecular weight determinations is ± 12%.

A solution of protein prep, 30 mg in 2 ml of buffer, was applied to the column and cocoa protein molecular weights were estimated from the standard curve.

RESULTS AND DISCUSSION

Extraction of Cocoa Proteins. Attempts to extract protein from cocoa beans with water, dilute salt solutions, and dilute acid or base resulted in a maximum recovery of 25% of the protein nitrogen. Moreover, extracts browned rapidly and formed insoluble precipitates, even when refrigerated.

Of the methods evaluated, the procedure of Meredith and Wren (1966) proved best for extraction of protein in highest yield and with the least amount of extraneous material. Urea and hexadecyltrimethylammonium bromide aid solubilization of proteins by disrupting hydrophilic and hydrophobic bonds, respectively. The sonication step adopted for the cocoa study speeded and improved protein recovery by enhancing penetration of cocoa particles.

The problem with discoloration and protein insolubilization is believed caused primarily by residual polyphenolic material not removed by methanol extraction. Polyphenols can oxidize to quinones, which may form covalent bonds with proteins to render them insoluble. Ionization of phenolic hydrogen results in more easily oxidized polyphenols (Loomis, 1969). Since low pH prevents this dissociation, adoption of acidic conditions in the buffer for extracting protein should favor a more stable protein extract, and this proved to be the case.

An acidic condition in the buffer was also necessary to make PolyClar AT effective in absorbing polyphenols and

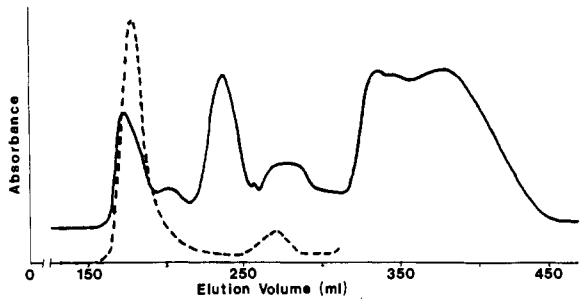

Figure 1. Typical elution curve of cocoa protein extract from Sephadex LH20 column: (—) column elution monitor at 280 nm; (- - -) protein by Lowry's method, absorbance at 750 nm.

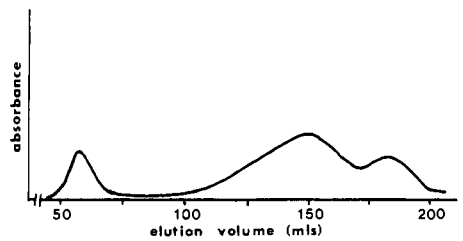

Figure 2. Elution curve of extracted cocoa proteins from a calibrated Sephadex G200 column used for estimating molecular weights.

tannins. PolyClar AT, a nonsoluble form of polyvinylpyrrolidone, functions only in nonionized form. Although buffers above pH 7 have been commonly used, acidic conditions are preferred when polyphenols may be a problem.

Ascorbic acid and EDTA in the protein extracting buffer are also important. They act as antioxidants to favor reduction of quinones and the inhibition of polyphenol oxidase (Pierpoint, 1966). Protein extracts without these antioxidants were much darker. Although Quesnel and Jugmohunsingh (1970) indicated EDTA at concentrations of 2×10^{-4} M had no effect on polyphenol oxidase activity, better quality protein extracts were, nevertheless, obtained in the study reported herein. EDTA proved more effective than diethyldithiocarbamate.

Sephadex LH20 separated protein from nonprotein components as shown in Figure 1. LH20, a modified form of G25 having the same molecular weight fractionating range, was selected primarily because it has a slight affinity for hydroxyl groups. High molecular weight polyphenols and proteins could, thus, be more satisfactorily resolved.

Two peaks in Figure 1 were Lowry positive, the first being protein while the second at 260 ml elution volume probably was peptide. Lowry determinations were terminated at 310 ml due to the formation of a precipitate upon addition of Folin phenol reagent. This was not considered a problem worthy of solution, since all protein should have passed through the column at this point.

Protein, peptide, and free amino acids contribute to summed amino acid values as determined on hydrolyzed samples, with the former predominating. Summed values were, therefore, considered a reasonable measure of extractable protein, and were used as the criterion of extraction efficiency and precision. Three lots of sample UF 667, extracted and analyzed according to the described method, yielded recoveries of 71.0, 72.2, and 70.5% of total cocoa bean amino acids in the protein fraction. An alternate route would have been Kjeldahl nitrogen measurements which would have necessitated an additional analysis for alkaloids, a major nitrogen contributor.

Table I. Amino Acid Patterns for Hydrolyzed Fat-Free Mass from Various Types of Pigmented and Nonpigmented Cocoa Beans

	Nonpigmented, mol %			Pigmented, mol %		
Amino acid	Porcelana	Criollo	Catongo	Nacional	UF 667	Pound 7
Lys	5.33	4.33	5.11	5.50	5.09	5.44
His	1.79	1.42	1.62	1.82	1.58	1.56
Arg	5.35	4.52	4.98	5.71	4.96	5.24
Asp	13.75	14.66	12.95	12.55	13.63	12.61
Thr	5.15	5.18	4.70	4.92	4.49	4.80
Ser	6.54	6.61	5.82	6.18	5.19	6.37
Glu	12.80	14.89	14.94	15.50	15.39	16.03
Pro	5.98	4.99	4.87	4.22	4.83	5.22
Gly	8.50	8.62	9.21	8.06	8.86	9.06
Ala	7.08	7.34	7.38	7.22	7.67	7.66
Cys	1.75	1.71	1.88	1.81	1.78	1.99
Val	7.57	7.68	7.95	7.60	7.83	8.03
Met	0.96	0.98	0.93	0.88	1.04	1.04
Ile	4.09	4.09	4.19	4.10	4.21	4.22
Leu	6.74	6.81	6.79	6.75	6.69	6.85
Tyr	2.48	2.17	2.28	2.52	2.39	2.45
Phe	4.07	4.03	4.42	4.58	4.39	4.44

Table II. Total Protein, Extractable Protein, and Purity of Protein Preps of Several Varieties of Cocoa Beans[a]

Cocoa bean variety	Total protein,[b] mg/g	Extractable protein		Protein prep purity, %[e]
		mg/g[c]	%[d]	
Nonpigmented				
Porcelana	206	66	32	31
Criollo	237	78	33	24
Catongo	268	89	33	44
Pigmented				
Nacional	268	186	70	53
UF 667	310	220	71	62
Pound 7	315	194	62	66

[a] Defatted bean mass basis. [b] From amino acid analysis of hydrolyzed defatted bean mass. [c] From Lowry analysis of protein fraction from Sephadex LH20 column. [d] As percent of total protein amino acids. [e] From amino acid analysis of protein prep.

Molecular Weight Estimation. A rough approximation of molecular weight ranges for cocoa protein was obtained through comparison with elution volumes of proteins of known molecular weights on a calibrated Sephadex G200 column. Data for individual protein preps were not collected; rather, several preps were pooled and analyzed to obtain an average of values. Cocoa protein gave three major peaks (Figure 2), one at 62 ml, the largest peak at 149 ml, and a peak of intermediate size at 185 ml elution volume. The slope of the largest peak suggested several proteins eluting between 100 and 150 ml corresponding to molecular weights between 45000 and 14000. The three peaks represent grouped molecular weights of 132000, 14400, and 5500 with most being in the 14400 class.

Comparisons among Varieties. *Protein Contents and Prep Purities.* Since only about 3% of the amino acids in cocoa beans are free (Maravalhas, 1972), analysis of acid-hydrolyzed, fat-free cocoa mass yields amino acid patterns which reflect protein composition. As shown in Table I, discernible differences among nonpigmented and pigmented varieties are not evident. Compositions, expressed as mole percent of each amino acid, were similar and in reasonable agreement with data for other types of beans (Wolf, 1958; Maravalhas, 1972).

Conversion from molar to mass data yields a summed value of amino acids which essentially is the protein concentration in cocoa beans (Maravalhas, 1972). When the amino acid data in Table I are thus applied, differences

Table III. Solubility Characteristics in Different Solvents of Protein Preps from Several Varieties of Cocoa Beans

Cocoa bean variety	Percentage distribution			
	Albumin	Globulin	Prolamine	Glutelin
Nonpigmented				
Porcelana	33	19	11	37
Criollo	32	25	12	31
Catongo	37	19	13	30
Pigmented				
Nacional	51	25	12	12
UF 667	71	2	17	10
Pound 7	71	1	20	8

in protein contents among varieties are revealed (Table II). Total protein for pigmented varieties, 268 to 315 mg/g of defatted bean, averaged 25% greater than in nonpigmented beans. This relationship has been observed previously with the suggestion that it may be a key to understanding why Forastero varieties require longer fermentation periods than Criollo (Roelofsen, 1958). This matter warrants more intensive study through investigations of additional cocoas of authenticated origins.

Of particular interest and importance are differences in extractable protein and protein prep purity. Extractable protein is the amount in cocoa beans solubilized by the extracting acid–urea–detergent solution. Protein prep is the freeze dehydrated protein fraction with most of the extraction solution and extraneous substances removed. Prep purity determined by amino acid analysis reflects the amount of covalently bonded nonprotein material.

As shown in Table II only about one-third of the protein content of nonpigmented beans could be solubilized and contribute to extractable protein values; more than twice as much could be extracted from pigmented varieties. Moreover, only one-third of the prep material from nonpigmented beans was protein; for pigmented beans it was 53–66%. As will be discussed, tanning reactions of polyphenols most likely contribute to the differences.

Solubility Classification. Contrasts are also evident in the solubility data for protein preps in water (albumin), sodium chloride (globulin), aqueous ethanol (prolamine), and dilute base (glutelin). Table III reveals pigmented varieties as being more easily solubilized than nonpigmented beans. This is consistent with Table II showing higher extractable protein values and preps of greater purity for pigmented varieties.

Disc Gel Electrophoresis. Separations obtained for Fluram marked protein preps by disc gel electrophoresis are presented in Figure 3. Protein bands appear between regions A and D with the greatest concentration being between A and C. The intense band at D is not protein. It is urea from the protein recovery procedure. Urea's presence is fortuitous since it serves as a marker. The three samples on the left are pigmented varieties UF 667, Pound 7, and Nacional. They show eight protein bands. This is in contrast to the protein preps from nonpigmented Porcelana, Catongo, and especially Criollo which separated into fewer and less intense bands. Electrophoresis results are compatible with data in Tables II and III.

Although the same amount of protein prep was applied to each gel, actual protein amounts were different due to the variable purity of the prep material (Table II). Thus, on an equal weight basis only about half as much protein was subjected to separation in the case of nonpigmented beans, and a decrease in number and clarity of bands might be expected. However, the high sensitivity of the Fluram method (Zak and Keeney, 1974) indicates that the magnitude of differences in protein amounts would not materially influence results obtained. Patterns in Figure 3 reflect actual differences among varieties of cocoa beans.

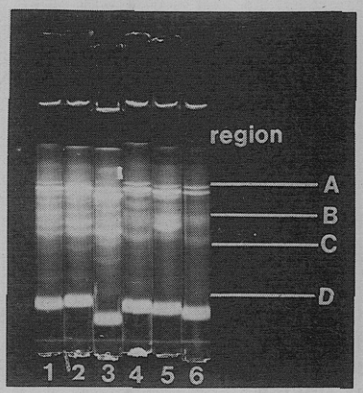

Figure 3. Disc gel electrophoresis of Fluram-treated protein preps: (1) UF667, (2) Pound 7, (3) Nacional, (4) Porcelana, (5) Catongo, (6) Criollo. Most of protein in regions A–C. Intense band at D is urea from buffer.

As explained more fully in a complementary report (Zak and Keeney, 1976), difficulties encountered in developing a protein extraction procedure, the incomplete recoveries realized, and the impure nature of protein preps finally obtained reflect the influences of cocoa–polyphenol interactions. Reasons for the profound differences between pigmented and nonpigmented varieties of cocoa beans are less clear and can be discussed only in a speculative sense. The only obvious compositional difference, other than protein content, is the presence of anthocyanidins in the polyphenol fraction of pigmented beans. Whether the relationship is casual or anthocyanidins actually do affect protein solubility cannot be resolved without further work.

Important events during fermentation which enhance desirable quality attributes are oxidation of polyphenols and subsequent complexing with protein. Criollo beans require only a 2- or 3-day fermentation, whereas Forastero beans, UF 667 and Pound 7, need longer fermentation periods (4–6 days). If insolubilization of protein is a critical factor, then a short fermentation for nonpigmented Criollo is a logical consequence of the already formed polyphenol–protein complex as reflected in protein recovery and prep purity data in Table II, the solubility results in Table III, and to a lesser degree disc gel electrophoresis patterns shown in Figure 3. Protein characterization data for Catongo and Nacional (Arriba) beans are somewhat intermediate between Criollo and the true Forastero types as are fermentation requirements. Traditionally, Nacional beans are lightly fermented.

A combination of the analyses herein described is suggested as an approach to classification of beans of unknown origin, a guide in plant breeding programs, and a systematic way to study complex interactions of protein occurring during fermentation, drying, and further processing of cocoa beans.

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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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