Extraction, Fractionation, and Amino Acid Composition of Brazilian Comun Cacao Proteins

Proteins were extracted from the seeds of Brazilian Comun cacao with a urea buffer and purified on a Sephadex G-25 column. The protein extract was then separated into eight groups using stepwise changes in pH on a Sephadex SP-25 cation exchange column. Each protein group was significantly different in amino acid composition from every other group. Based on this method, 13 amino acids should be considered when classifying various cacaos according to genetic origins. Glutamic acid, alanine, and arginine were most variable. Least likely to reflect differences among protein groups were lysine, histidine, cysteine, and methionine.

Cacao protein, 11–13% of dry bean weight, is recognized as an important factor influencing the characteristics of chocolate, especially flavor. Chemical indices related to proteins, such as Kjeldahl nitrogen and amino acid analyses, have been studied by numerous investigators including Bracco et al. (1969), Maravalhas (1972), and Reineccius et al. (1972).

Recently, Zak and Keeney (1976) reported extraction procedures for recovering cacao proteins in relatively pure form. Previous attempts (DeWitt, 1957; Neipage, 1961) resulted in extensive tanning which masked protein differences among cacao varieties and made characterization and fractionation of the native proteins difficult. The research reported herein was undertaken to further refine methods so that subtle differences related to variety might be revealed.

MATERIALS AND METHODS

Source of Samples. Brazilian Comun cacao beans were obtained in their pods from the Cacao Research Center, Itabuna, Bahia, Brazil. Three days after harvesting, the pods arrived at University Park and were immediately frozen and maintained in the frozen state until analyzed.

Preparation of Beans for Extraction. Frozen cacao pods were cut open and, after removal of the husk and mucilagenous pulp, the beans were freeze dehydrated. Vacuum was broken with nitrogen gas. Dried beans were pulverized to a fine powder in a CRC (Chemical Rubber Co., Cleveland, Ohio) mini mill. Small pieces of dry ice were ground with the beans to prevent frictional heat of grinding from melting the cacao lipids.

Protein Extraction. A modification of the method of Zak and Keeney (1976) was developed to extract the cacao proteins. Four 1-g samples of finely ground cacao beans were each extracted 3 times in a 15-ml centrifuge tube with 10 ml of petroleum ether. Following centrifugation, the solvent and dissolved lipid material were decanted and discarded.

Polyphenolic material was extracted from defatted pellets with 80% aqueous acetone containing 0.01 M ascorbic acid. The pellets were resuspended in 10 ml of acetone and centrifuged, and the supernatant was decanted and discarded. This extraction process was repeated three times.

Protein was recovered from the residual material by sonication for 10 min with 10 ml of a solution containing 0.0167 M citric acid, 3.5 M urea, 0.01 M ascorbic acid, 0.01 M hexadecyltrimethylammonium bromide, and 2% EDTA (sodium form). After centrifugation the supernatants from the four samples were pooled and filtered through Whatman No. 1 paper.

The total filtrate, 36–39 ml, was applied to a 2.5 × 100 cm Sephadex G-25 medium (Pharmacia Fine Chemicals, Inc.) column and eluted with a urea–citric acid solution (UC buffer) containing 0.0167 M citric acid, 3.5 M urea, and 0.1 M NaCl, with pH adjusted to 3.10 ± 0.05. This was pumped through the column at a rate of 2.0–2.2 ml/min with a variable speed peristaltic pump.

The eluent from the column was monitored at 280 nm with an Isco Model UA-4 detector (Instrumentation Specialties Co.) and strip chart recorder. Protein concentration in the first peak, 75–80 ml, was measured by the method of Lowry et al. (1951) using papain as a standard. All values reported in this study are based on Lowry papain equivalents.

Protein Fractionation. The protein fraction (peak no. 1, Figure 1), less 10 ml needed for quantitation and amino acid analyses, was applied to a 1.5 × 30 cm Sephadex SP-25 cation exchange column, previously equilibrated with 150 ml of UC buffer pumped through the column at a rate of 2.0–2.2 ml/min. After sample application the column was eluted with buffers of increasing pH (3.10, 3.50, 4.00, 4.50, 5.00, and 7.00). Buffer volume at each pH was 50 ml, except at pH 3.5 when 60 ml was applied. The column was finally eluted with 50 ml of 0.1 M NaOH. Each buffer had been adjusted to the desired pH by titrating the UC buffer with a solution containing 0.0167 M sodium citrate, 3.5 M urea, and 0.1 M NaCl. End points were determined with a digital pH meter. Eluent from the cation exchange column was monitored at 280 nm as previously described (Figure 2) and contents of tubes from the fraction collector representing each peak were combined.

After removal of 1-ml aliquots for protein quantitation by the Lowry method, each protein fraction was dialyzed 48 h against running tap water followed by 24 h against distilled water. The dialyzate was then taken to dryness under reduced pressure on a rotary evaporator.

A brown band remained at the top of the SP-25 column, even after elution with 0.1 N NaOH. This band material was cut from the column and rinsed with distilled water to remove residual buffer. This is protein group 8 in Table I.

Amino Acid Analysis. Dried protein fractions were dissolved in 6.0 ml of 6 N HCl, sealed in evacuated ampules (Wheaton Glass No. 12310), and held 24 h at 110 °C. Following hydrolysis, contents were filtered and taken to dryness under reduced pressure. Distilled water, 10–15 ml, was added and the samples were taken to dryness a second time. The brown band material from the ion exchange column was similarly treated.

Samples were diluted to yield a final protein hydrolysate concentration of 0.25 mg/ml. Charge to a Beckman Model 120C Autoanalyzer was 1 ml of hydrolysate solution to PA 35 and AA15 resins for the basic and acidic plus neutral amino acids, respectively.

Amino acid analyses, in triplicate, were carried out on protein fractions corresponding to the seven peaks from

Table I. Group Differences in Amino Acid Composition (Mole Percent) of Brazilian Comun Cacao Proteins Fractionated on Sephadex SP-25 Ion Exchange Resin

	Protein group[a]							
	1	2	3	4	5	6	7	8[b]
Lys	3.6^{bc}	4.3^{abc}	3.0^{c}	4.6^{abc}	4.1^{abc}	5.8^{a}	4.1^{abc}	5.6^{ab}
His	0.8^{a}	0.7^{a}	0.6^{a}	0.5^{a}	0.5^{a}	1.2^{a}	1.0^{a}	1.0^{a}
Arg	2.3^{e}	2.0^{e}	3.7^{d}	5.7^{c}	9.8^{a}	6.9^{b}	5.2^{c}	5.5^{c}
Asp	11.9^{c}	17.1^{a}	16.3^{ab}	16.3^{ab}	8.6^{e}	10.9^{d}	14.8^{b}	14.7^{b}
Thr	8.2^{b}	9.4^{a}	5.4^{d}	6.4^{d}	4.0^{f}	4.6^{ef}	6.9^{cd}	7.3^{c}
Ser	11.8^{a}	4.6^{e}	7.6^{c}	9.2^{b}	5.3^{e}	6.5^{d}	7.9^{c}	9.1^{b}
Glu	9.4^{d}	9.5^{d}	12.7^{c}	8.3^{d}	37.0^{a}	23.8^{b}	9.1^{d}	5.0^{e}
Pro	5.2^{abc}	6.2^{a}	5.9^{ab}	4.9^{bc}	3.8^{c}	4.7^{bc}	5.2^{abc}	4.6^{bc}
Gly	11.7^{b}	13.2^{a}	9.5^{d}	10.8^{bc}	7.3^{e}	8.0^{e}	10.6^{cd}	10.3^{cd}
Ala	11.1^{a}	8.0^{b}	8.2^{b}	6.5^{c}	4.1^{d}	4.8^{d}	6.9^{c}	7.0^{c}
Cys	a	Tr^{a}	0.6^{a}	a	a	Tr^{a}	a	Tr^{a}
Val	5.6^{c}	8.2^{b}	9.4^{a}	8.9^{ab}	4.1^{d}	5.7^{c}	8.8^{ab}	7.0^{b}
Met	a	Tr^{a}	Tr^{a}	Tr^{a}	a	0.7^{a}	a	a
Ile	4.4^{bc}	4.0^{cd}	3.5^{de}	4.4^{c}	2.4^{f}	3.1^{e}	4.6^{ab}	5.2^{a}
Leu	8.2^{abc}	6.5^{e}	7.4^{cde}	7.9^{bcd}	5.0^{f}	7.1^{de}	8.5^{ab}	9.0^{a}
Tyr	2.8^{a}	2.7^{a}	1.1^{c}	1.7^{bc}	2.3^{ab}	2.7^{a}	2.2^{ab}	1.6^{bc}
Phe	2.9^{c}	3.6^{b}	6.2^{a}	4.0^{bc}	2.2^{e}	2.5^{de}	4.2^{b}	4.3^{b}

[a] Amino acid values in the same row with a common roman superscript are not different ($p < 0.05$). [b] Obtained from the brown proteinaceous material which could not be eluted from Sephadex SP-25 ion exchange column with base, acid, or salt.

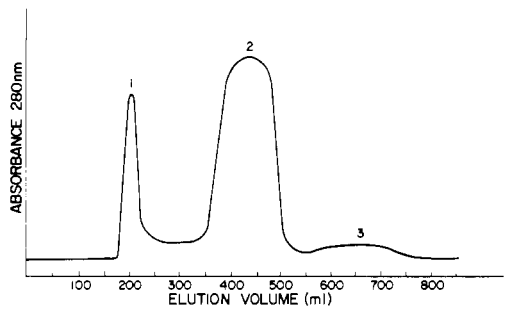

Figure 1. Typical elution profile of cacao protein extract from Sephadex G-25 medium column.

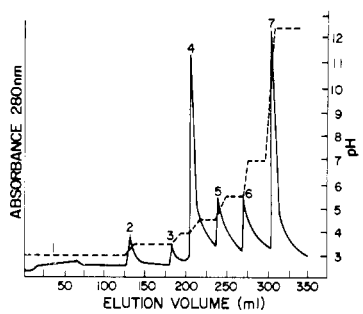

Figure 2. Elution curve of purified cacao proteins eluted with stepwise changes in pH from a column of Sephadex SP-25 cation exchange resin.

the ion exchange column, the recovered brown band material which did not elute, and a sample from the total extractable protein material taken before separation. Statistical analyses were performed with the aid of a computer. Analysis of variance and Duncan's (Bayesian) least significant difference test (DLSD) were executed using library programs available at the Computation Center of the Pennsylvania State University.

RESULTS AND DISCUSSION

Extraction of Cacao Proteins. The extraction procedure adopted for this investigation was directed toward the recovery of a stable protein fraction containing the least amount of extraneous material. Based on protein nitrogen values and amino acid analyses, 38–43% of the protein content of cacao was extracted. Deviations from the procedure outlined by Zak and Keeney (1976) were instituted to minimize polyphenolic oxidation and to increase the compatability of the system with the properties of the SP-25 cation exchange resin. Aqueous acetone with ascorbic acid was found to be more effective than anhydrous methanol in inhibiting the browning of extractable cacao proteins. This was probably due to a more complete removal of polyphenols (Loomis, 1969). Citric acid, substituted for acetic acid, resulted in greater buffering capacities both at low pH and over a greater pH range. NaCl was found necessary to maintain the counterion equilibrium of the SP-25 resin. Without NaCl in the buffer solution the ion exchange column could not be equilibrated at pH 3.10.

Table II. Amino Acids in Cacao Protein Groups Which Were Different from the Remaining Protein Groups

Protein group	Amino acid
1	Thr, Ser, Ala
2	Thr, Gly
3	Arg, Glu, Phe
4	
5	Arg, Asp, Glu, Val, Ile, Leu
6	Arg, Thr, Ser
7	
8	Glu

Fractionation of Cacao Proteins. Of the protein material applied to the SP-25 column, 70–80% was recovered in peaks labeled 1–7 in Figure 2. Quantitation of protein fractions, 1 through 7, by the method of Lowry accounted for 3.6, 4.1, 4.3, 22.3, 16.4, 23.0, and 26.5% of the protein, respectively.

Elution with 0.1 M NaOH, 0.1 M HCl, or 1.0 M NaCl failed to remove the brown band, previously referred to. These conditions should have eliminated ionic binding between the protein and ion exchange resin. Binding of the pigmented material to the column may possibly be due to covalent linkages with the resin via contaminating polyphenols or resin encapsulation by protein through extensive polyphenol–protein polymerization. Amino acid analysis of the brown pigment (group 8, Table I) confirmed the proteinaceous nature of this material.

Disc gel electrophoresis of cacao proteins by Zak and Keeney (1976) revealed 9–12 major protein bands along with several minor ones. For this reason, protein fractions obtained in the current study are referred to as protein groups having similar charge characteristics rather than as homogeneous proteins.

Table III. Ranking of Amino Acids in Order of Decreasing Variability Based on F Ratios Obtained from the Analysis of Variance of the Cacao Protein Groups

Ranking order	Amino acid	F ratio
1	Glu	87.59
2	Ala	60.20
3	Arg	55.96
4	Phe	36.97
5	Ser	31.13
6	Val	26.71
7	Thr	24.90
8	Asp	20.37
9	Ile	20.22
10	Gly	18.43
11	Leu	9.25
12	Tyr	4.61
13	Pro	2.94
	$p < 0.05$	
14	Lys	2.52
15	His	1.43
16	Cys	1.06
17	Met	0.41

Amino Acid Analyses. Statistical analyses using Duncan's least significant difference test ($p < 0.05$) showed all protein groups to be different with respect to amino acid composition. Six groups had at least one amino acid significantly different in mole percent when compared to every other group (Table II). Group 5 had 6 amino acids in this category. This may reflect its unusually high glutamic acid content. Neither group 4 nor 7 had an amino acid at a level which was significantly different from all other groups. These two groups were quite similar, varying only in serine and isoleucine contents. Histidine and especially cysteine and methionine were present only in trace amounts in all instances.

In Table III the amino acids are ranked in descending order based on F ratios. A high F ratio indicates a wide distribution for the mole percent of an amino acid among protein groups, while taking into consideration the effect of experimental error on the distribution. Based on this method, 13 amino acids vary sufficiently to warrant further study, since there is a 95% chance that the mole percent of at least one of them in a protein group will be different than the levels in the remaining groups. If the p value of the F ratio were decreased to 0.005, differences would still be expected for 12 of the amino acids in Table III. Lysine, histidine, cysteine, and methionine have F ratios below the value required for $p < 0.05$, and it is unlikely that any real differences between groups would be found.

Essentially all of the cacao grown throughout tropical regions of the world evolved from two types, Criollo and Forastaro, both of which are indigenous to and about the western Amazon basin. Protein content of the latter is higher, and recovered protein fractions are less contaminated and more soluble compared to Criollo (Zak and Keeney, 1976). These differences reflect in part protein reactions with oxidized polyphenols.

It would seem that differences in amino acid profiles between Criollo and Forastaro would also be involved in explaining why Criollo is more easily and extensively tanned, and why electrophoresis patterns are not similar. However, the data of Zak and Keeney (1976) are inconclusive in this regard. Improvements in methodology described herein, especially the introduction of ion exchange chromatography on Sephadex SP-25, resulted in the recovery of several protein fractions from a single cacao type, and with an amino acid pattern different from the other groups. Using these methods we anticipate that amino acid differences among protein groups of Criollo and Forastaro Cacao and varieties derived from them will be found. Hopefully, this information might serve as a chemical index in evaluating wild genotypes and classifying cacao types currently in use.

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Determination of Biphenyl by Gas and Liquid Chromatography

Citrus fruits in shipping cartons are protected from decay with biphenyl-impregnated pads. Two methods of analyzing the biphenyl content of the pads, by gas and liquid chromatography, were compared and both were satisfactory. Analysis of extracts of whole pads rather than of representative areas is suggested. A method of analysis of biphenyl vapor by gas chromatography was developed. This entailed multiple injections from a heated sampling syringe to overcome the tendency of biphenyl to adhere to the glass. This method should be useful in determining relationships between vapor concentration and fungistatic activity and to atmospheric monitoring.

For the analysis of biphenyl, a widely used fungistatic agent, infrared (Newhall et al., 1954), ultraviolet following cleanup by thin-layer chromatography (Norman et al., 1966, 1968, 1969), gas chromatography of solutions (Beernaert, 1973; Morries, 1973; Wells et al., 1963), and liquid chromatography of residues in citrus products (Reeder, 1975) and of vapors sampled with a loop (Sharma and Palmer, 1974) have been used. Within cartons of packed citrus fruit, biphenyl is usually applied by insertion of two kraft paper pads impregnated with the fungistat