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Walter Rudzinski*[3]
Luis Echegoyen[1]
William Jobin[2]

[1]Department of Chemistry
University of Puerto Rico
Rio Piedras, Puerto Rico 00931
[2]Center of Energy and Environment Research
Caparra Heights Station
San Juan, Puerto Rico 00935
[3]Present address: Department of Chemistry
Southwest Texas State University
San Marcos, Texas 78666

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Comparison of Several Types of Cocoa Beans Relative to Fractionated Protein Components

Protein of seed cotyledons in fruit samples from the clonal collection of CATIE, Turrialba, Costa Rica, were extracted into urea buffer, purified on a column of Sephadex G-25, and separated into seven protein groups on a column of SP-25, cation-exchange resin. Among ten samples of Criollo, Trinitario, Nacional, and Forastero cocoa beans, statistically significant ($P \leq 0.01$) differences were found in total and extractable protein contents, in protein group profiles, and in amino acid patterns. Although differences were found among known types of cocoa, they were not considered great enough to suggest the procedures of this investigation as a practical approach to classifying cocoa of unknown genetic origin.

From an evolutionary viewpoint, commercial cocoa has developed from two types of beans, Criollo and Forastero of the species *Theobroma cacao*. Indigenous to the Amazon River basin, cacao has been transplanted to other tropical regions over the past century, most notably West Central Africa. Natural adaptation and breeding programs, directed toward disease resistance and increased production, now yield genetically heterogeneous commercial crops. Production has not kept pace with increasing world demand, and improved, high-yielding seed material is constantly sought. Evaluation of wild genotypes and the products of commercialization require reliable methods of characterization. Protein, which constitutes about 10% of bean dry weight, is a logical component for study.

Using a cation-exchange resin column, protein extract from Brazilian comum cocoa seed was separated into eight groups by stepwise change in pH (Timbie and Keeney, 1977). Each protein group was significantly different in amino acid composition from every other group. Thirteen amino acids were involved in this variability. Reported herein are results of protein analyses of several cocoa bean samples of known origins selected to test this approach to cacao classification.

MATERIALS AND METHODS

Samples. Cocoa fruit of authenticated genetic origin was selected by J. Soria from clonal collection of CATIE, Turrialba, Costa Rica. Varietal types were Criollo, Trinitario (hybrids of Criollo and Forastero), Nacional, and Amazon Forastero. Beans and adhering pulp material were removed and freeze dehydrated within 48 h after harvest of the fruit. The testa material was peeled away, and the dry cotyledon portion was stored in an evacuated desicator.

Fat, Total Protein, and Amino Acid Analyses. Dry cotyledons (25 g) were pulverized to a fine powder in a grinding mill, small pieces of dry ice being added to prevent melting of cocoa lipids. Soxhlet extraction with petroleum ether (18 h) yielded a defatted residue, which was ground a second time, after vacuum treatment to remove residual ether. Fat content of the cotyledons was determined by evaporating the petroleum ether fraction to constant weight. Alkaloid nitrogen [from theobromine and caffeine analysis according to Timbie and Keeney (1978)], subtracted from Kjeldahl nitrogen (Pregal and Fyleman, 1927) yielded protein nitrogen in the nonfat cocoa residue. This difference × 6.25 was crude protein. Defatted cotyledon powders and various protein fractions were hydrolyzed in 6 N hydrochloric acid and subjected to amino acid analysis (Timbie and Keeney, 1977). Amino protein values were calculated from these data.

Protein Extraction, Fractionation, and Analysis. The procedures followed were those described by Timbie and Keeney (1977). Polyphenols were removed from defatted cocoa powder by acetone extraction, before treatment with protein-solubilizing buffer. The protein extract was purified on a column of Sephadex G-25 medium (Pharmacia Fine Chemicals, Inc.). Protein was then fractionated, by stepwise increase of eluting buffer pH, on a 0.9 × 20 cm column of Sephadex SP-25 cation-exchange resin. An aliquot of each protein peak volume was used for quantitative protein determination (UV absorbance at 280 nm); the remaining portion was further purified by dialysis, before hydrolysis and amino acid analysis.

Statistical Analyses. Variances were calculated using the library program ANOVES/ANOVUM (Computation Center, The Pennsylvania State University). All mean separations were calculated for $P \leq 0.01$ using Duncan's

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Table I. Protein Content of Cocoa Bean Cotyledons from Fruit Representing Different Clones of *Theobroma cacao*[a]

clone	varietal type	dry, whole cotyledons[b] fat, %	dry, whole cotyledons[b] crude protein,[c] %	dry, whole cotyledons[b] amino protein,[d] %	fat-free solids crude protein,[c] %	fat-free solids amino protein,[d] %
NIC-79	Criollo	39.5e	10.4	10.0d	17.3	16.6c
PENT × MAT	Trinitario	49.9c	8.9	8.8c	17.8	17.6c
UF-11	Trinitario	51.0b	10.1	10.1d	20.5	20.6b
UF-667	Trinitario	50.5c	11.3	10.0d	22.9	20.1b
UF-296	Trinitario	53.3a	12.3	10.7bc	26.2	22.9a
N22A3	Nacional	53.8a	11.2	11.5a	24.2	23.2a
NA2233	Nacional	51.8b	11.8	11.2ab	24.4	23.4a
EEG-29	Forastero	48.2d	10.3	10.4cd	19.9	20.2b
EEG-64	Forastero	48.1d	11.6	10.2cd	22.4	19.8b
SIC-250	Forastero	51.9b	11.3	11.4a	23.5	23.7a
av, 10 samples		49.8	11.0	10.4	21.9	20.8

[a] Values followed by a common letter are not significantly different ($P \leq 0.01$). [b] Freeze dehydrated immediately after opening of pod and removal of testa. [c] (Crude protein N − alkaloid N)6.25. [d] Sum of amino acids (g/100 g) from analysis of protein hydrolyzate.

(Bayesian) least significant difference test.

RESULTS AND DISCUSSIONS

For individual samples, the value for crude protein (adjusted for alkaloids) was similar to that based on amino protein, although, some of the latter tended to be slightly lower (Table I). Probably this reflects destruction of a portion of certain amino acids during acid hydrolysis. Generally, protein contents shown in Table I are consistent with those reported by others (Roesch et al., 1959; Neipage, 1961; Zak and Keeney, 1976). Precise comparisons are difficult since some investigators failed to adequately describe cocoa samples and their treatments.

Differences in amino protein content among cotyledons from the ten clones were statistically significant ($P \leq 0.01$). PENT × MAT contained the least amount of amino protein (8.8%), and N22A3 contained the highest amount (11.5%). Although whole cotyledons could not be clearly segregated according to protein content, three distinct groupings can be made on a defatted cocoa solids basis (low, NIC-79 and PENT × MAT; intermediate, UF-11, UF-667, EEG-29, EEG-64; high, UF-296, N22A3, NA2233, SIC-250). Inability to distinguish among groups of whole cotyledons is attributed to the wide variation found in fat content (39.5% in NIC-79 to 53.8% fat in N22A3). The trend in amino protein content among Trinitario clones deserves mention. J. Soria's rating of Criollo dominance placed PENT × MAT closest to Criollo, followed in order by UF-11, UF-667, and UF-296. Table I shows an inverse relationship between Criollo dominance and amino protein content among defatted samples.

Amino acid profiles for cocoa protein from the clones are presented in Table II. Although pronounced compositional variations are not evident, statistical analysis revealed the involvement of 13 amino acids in significant differences between two or more clones. Nearly half of these differences were within the Trinitario clones. This is not unexpected, since Trinitarios are genetically less pure than Criollo, Nacional, and Forastero varieties.

Extractable protein (percentage of total protein extracted by buffer) among the ten varietal types varied from 39% for Criollo to 62% for Nacional. Trinitario and Forastero were intermediate, averaging 52% and 55% extractable protein, respectively. Zak and Keeney (1976) reported similar results. Reasons for the solubility differences are unclear. All samples were treated in an identical manner, and phenolic tannins should not be involved, unless the type and amount of polyphenols in the samples were variables. Nonpigmented Criollo beans do not contain the purple anthocyanins which characterize most varieties of cocoa beans, and other polyphenol differences have been reported (Forsyth and Quesnel, 1963). Thus, complexing of polyphenols to protein may be a factor in the solubility differences observed in this study.

Zak and Keeney (1976) found that protein purity, in terms of amino acid content, was substantially less in Criollo beans than in pigmented Forastero types. Therefore, solubility differences among clones may be due to inherent structural differences in the proteins, as well as to deterioration involving polyphenols. Native protein needs to be studied in greater detail, especially the characterization of the nonamino portion.

Amino acid patterns for protein recovered from the column of Sephadex G-25 were very similar for all cocoa types. However, striking differences are evident when they are compared to whole protein (amino acids in total hydrolyzate of nonfat cocoa solids). This indicates that some cocoa proteins are relatively insoluble, even in the urea buffer used. The most pronounced difference was in the NIC-79 clone; mole percent aspartic acid was 22% in the total hydrolyzate, but only 13% in the protein extract (serine, glutamic acid, and phenylalanine were involved

Table II. Amino Acid (Mole Percent) of Whole Cocoa Protein of Cotyledons Representing Different Clones[a,b]

amino acid	NIC-79	PENT × MAT	UF-11	UF-667	UF-296	N22A3	NA2233	EEG-29	EEG-64	SIC-250
Lys	5.59[a]	5.80[a]	6.01[a]	6.05[a]	6.13[a]	6.01[a]	6.11[a]	6.23[a]	7.06[a]	6.12[a]
His	2.07[ab]	1.76[ab]	1.88[ab]	1.63[b]	2.00[ab]	1.89[ab]	1.90[ab]	1.93[ab]	2.18[a]	1.92[ab]
Arg	4.20[b]	4.61[b]	5.18[ab]	5.02[ab]	5.20[ab]	5.35[ab]	5.23[ab]	4.92[ab]	6.08[a]	5.21[ab]
Asp	22.16[a]	15.09[bc]	15.51[b]	13.62[de]	13.56[d]	14.13[cde]	13.99[de]	14.11[de]	14.56[bcd]	14.45[cde]
Thr	4.18[a]	3.94[a]	4.92[a]	5.05[a]	5.01[a]	5.02[a]	4.67[a]	4.80[a]	5.03[a]	4.83[a]
Ser	5.32[e]	6.35[cd]	6.46[c]	6.44[c]	6.67[bc]	7.22[a]	6.33[cd]	6.39[c]	5.88[d]	6.98[ab]
Glu	13.08[d]	14.59[c]	14.78[c]	14.73[c]	16.39[a]	16.48[a]	16.45[a]	15.55[b]	14.75[c]	16.33[a]
Pro	5.03[a]	5.62[a]	5.27[a]	5.42[a]	5.23[a]	5.28[a]	5.39[a]	5.35[a]	5.48[a]	5.18[a]
Cys	0.00[b]	2.22[a]	0.00[b]	2.20[a]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00[b]
Gly	6.92[c]	8.53[a]	7.58[b]	8.37[a]	7.63[b]	7.29[bc]	7.47[b]	7.50[b]	8.22[a]	7.40[bc]
Ala	7.59[a]	6.90[b]	6.77[b]	6.78[b]	6.63[b]	6.80[b]	6.78[b]	6.48[b]	6.40[b]	6.77[b]
Val	6.57[a]	6.40[a]	6.68[a]	6.29[a]	6.52[a]	6.09[a]	6.83[a]	6.79[a]	5.86[a]	6.34[a]
Met	1.13[ab]	0.72[ef]	1.05[bc]	0.67[f]	0.94[cd]	0.85[de]	1.09[bc]	1.27[a]	0.75[ef]	1.04[bc]
Ile	3.72[b]	3.77[b]	3.78[b]	3.86[ab]	3.76[b]	3.45[c]	3.75[b]	4.00[a]	3.76[b]	3.52[c]
Leu	6.00[b]	6.68[a]	6.97[a]	6.85[a]	7.15[a]	7.06[a]	6.92[a]	7.19[a]	6.90[a]	6.92[a]
Tyr	2.46[b]	2.51[b]	2.61[ab]	2.63[ab]	2.84[a]	2.51[b]	2.67[ab]	2.87[a]	2.65[ab]	2.62[ab]
Phe	3.90[c]	4.40[ab]	4.45[ab]	4.25[b]	4.27[ab]	4.47[ab]	4.33[ab]	4.53[a]	4.32[ab]	4.26[b]

[a] Values in the same row followed by a common letter are not significantly different ($P \leq 0.01$). [b] On the basis of amino acid analysis of hydrolyzed nonfat cocoa solids.

Table III. Distribution of Cocoa Protein Fractions (g/100 g) Recovered from the SP-25 Cation-Exchange Column[a]

clone	protein peak 1	2	3	4	5	6	7
NIC-79	24.2[a]	7.6[a]	12.1[a]	16.9[a]	16.3[bc]	18.2[d]	4.7[abc]
PENT × MAT	23.2[ab]	3.8[e]	8.6[bc]	20.4[a]	17.8[b]	22.5[c]	3.7[bc]
UF-11	19.5[bcde]	5.3[d]	10.2[abc]	21.0[a]	17.3[bc]	23.2[c]	3.6[c]
UF-667	22.2[abc]	3.9[e]	5.3[d]	21.1[a]	15.6[bc]	28.0[a]	3.9[abc]
UF-296	11.2[f]	6.4[abcd]	8.0[cd]	20.3[a]	23.0[a]	25.2[abc]	5.9[abc]
N22A3	21.6[abcd]	5.5[cd]	7.3[cd]	17.5[a]	14.7[c]	27.1[ab]	6.3[a]
NA2233	18.1[de]	5.8[bcd]	7.6[cd]	18.6[a]	16.0[bc]	27.8[ab]	6.0[abc]
EEG-29	18.7[cde]	7.0[ab]	8.5[bc]	18.4[a]	16.1[bc]	25.0[bc]	6.2[ab]
EEG-64	20.9[abcd]	6.7[abc]	11.4[ab]	17.1[a]	16.2[bc]	23.0[c]	4.9[abc]
SIC-250	16.5[e]	5.5[cd]	10.0[abc]	19.7[a]	16.7[bc]	27.6[ab]	4.1[abc]

[a] Values in the same column followed by a common letter are not different ($P \leq 0.01$).

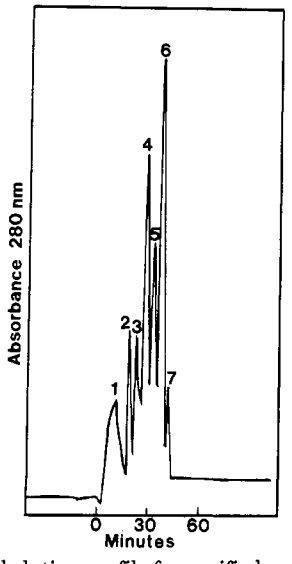

Figure 1. Typical elution profile for purified cocoa protein eluted from a column of Sephadex SP-25 cation-exchange resin. Protein groups 1–7 eluted successively with stepwise increased in buffer pH (3.10, 3.50, 4.00, 4.50, 5.00, and 7.00).

in the difference). A detailed presentation of data is available elsewhere (Timbie, 1977).

Protein recovered from the G-25 column was fractionated into seven protein groups on a column of SP-25 cation-exchange resin. Of the protein applied to the column, 88–96% was recovered in the eluate. A typical elution profile for fractionated cocoa protein is presented in Figure 1, and quantitative data for each of the 10 cocoa samples are listed in Table III.

Cocoa protein applied to the SP-25 cation-exchange column left a light-tan band at the top of the resin bed. Previously (Timbie and Keeney, 1977), much darker bands were encountered, intensity being correlated with phenolic tanning activity. This indicated that tanning reactions had been suppressed through the procedures followed in the trials reported herein.

From the study of Brazilian comum proteins (Timbie and Keeney, 1977), it had been anticipated that quantitative and qualitative differences among fractions from the cation-exchange resin column might prove useful in classifying cocoa of unknown genetic origin. Although significant differences attributable to bean type were found among protein groups (Table III), they are not great enough to have practical application. The same assessment holds for differences in amino acid profiles for each protein group among cocoa samples (Timbie, 1977).

CONCLUSIONS

Protein in cocoa cotyledons of authenticated genetic origin can be extracted and fractionated into protein groups to yield statistically significant differences among samples of cocoa beans. Methods used have value relative to an understanding of the chemistry of cocoa protein and its involvement in postharvest events which impact on quality attributes. However, this approach to cocoa classification does not appear to have practical applications.

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Douglas J. Timbie
Philip G. Keeney*

Department of Food Science
The Pennsylvania State University
Borland Laboratory
University Park, Pennsylvania 16802

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