Enzymatic Modification of the Extractability of Protein from Coconuts (*Cocos nucifera*)

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Enzymes capable of improving the extractability of protein from coconut flour from a control value of 50 to 60 to 85% have been derived from *Pestalotiopsis westerdijkii*. The resulting protein extracts appeared to be free of toxic products and to have a nutritive value equivalent to that of extracts prepared without enzyme treatment, but more extensive animal testing is needed. During enzyme treatment about half of the crude fiber was destroyed. The enzyme was produced in tray cultures on a coconut flour–Solka Floc support moistened with a mineral salts solution. After incubation for 10 to 11 days at 31–2° C., the enzyme was extracted with water, precipitated with $(NH_4)_2SO_4$, and dialyzed by gel filtration. For facilitating the extraction of protein from coconut flour optimum conditions were established at 40° C. for 3 hours at pH 5.6 to 6.0 using approximately 50 mg. of enzyme per gram of coconut residue.

Recent FAO (*6*) estimates of world copra production reach 3,320,000 metric tons per year. Geographically this production is centered in or near the same areas where protein deficiency is a chronic public health problem.

Although copra contains 20 to 25% protein of reasonably good nutritional quality, a number of situations combine to limit its consumption as food. High storage temperatures lead to rapid rancidification. In humid areas, large amounts are unfit for food use because of microbial contamination. In expeller-processed material excessive heating has frequently destroyed enough lysine to reduce the protein quality. The crude fiber levels of 10 to 12% reduce its usefulness in infant feeding. The interplay of all these problems results in wasting most of the supply, which represents a potential means of making an inroad on the world protein deficit.

The Central Food Technological Research Institute in Mysore and several groups in the Philippines have studied extraction of coconut protein extensively with the idea of using the aqueous extracts directly or of producing dry coconut protein powders as infant foods. Extraction efficiency, however, is generally low.

The present report demonstrates that the efficiency of protein extraction from coconut flour can be very significantly increased by treatment with fungal enzymes prior to extraction. Concomitantly with the improvement in protein extractability there is about a 50% reduction in the crude fiber content of the flour, so that it may be useful for infant feeding without extraction. One preliminary report of similar work using enzymes derived from *Trichoderma viride* has appeared (*13*).

Experimental Procedures

Mycological. The organisms used were from the U. S. Army Quartermaster collection in Natick, Mass. They included *Aspergillus terreus*, Q.M. 72f, *Basidiomycetes* sp., Q.M. 806, *Chaetomium globosum*, Q.M. 459, *Myrothecium verrucaria*, Q.M. 460, *Pestalotiopsis westerdijkii*, Q.M. 381, and *Trichoderma viride*, Q.M. 6a.

Culture media consisted of high quality coconut flour (Central Food Technology Research Laboratory, Mysore, India), Solka Floc (Brown Co., Berlin, N.H.), and the trace mineral salts solution of Reese and Mandels (*15*).

Tray cultures with a 2-cm. layer of moistened substrate were inoculated with a spore suspension and incubated at the temperatures indicated for the times specified in each experiment. After incubation the enzymes were extracted from the cultures as described by Toyama (*20*). Material soluble in 30% saturated $(NH_4)_2SO_4$ but not in 80% saturated $(NH_4)_2SO_4$ was redissolved in one tenth of the original volume of water and desalted by gel filtration using Sephadex-G-25.

Enzymological. The assays used were that of Hash and King (*8*) for aryl-β-glucosidase, carboxymethylcellulase, or C_x (*7*), and of Flora for hydrocellulase or C_1 (*5*).

Chemical. Proximate analyses were carried out as described by the AOAC (*1*). The procedure of Johnson (*10*) was used for micro-Kjeldahl analyses. Total sugars and their degree of polymerization were determined as described by Timell (*19*). The analysis of Nelson (*12*) as modified by Somogyi (*17*) was used for reducing sugars. The ethyl acetate–pyridine–water and the pyridine–butanol–water solvents of Jermyn and Isherwood (*9*) were used for paper chromatography along with the periodate–benzidine spray of Cifonelli and Smith (*3*). Individual oligosaccharides were analyzed as described by Cole and King (*4*).

For amino acid analyses duplicate samples were sealed under N_2 in glass tubes with 100 times their weight of 6*N* HCl and heated at 121° C. for 24, 48, or 72 hours. After analysis in duplicate as described by

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Spackman, Stein, and Moore (*18*), the values were corrected for losses during hydrolysis. Internal standards of norleucine and β-guanidinoalanine were used routinely with the long and the short columns, respectively.

Results

An initial comparision was made of the six actively cellulolytic fungi grown on coconut flour–trace mineral medium (Table I). Five grams of coconut flour plus 25 ml. of trace mineral solution were sterilized in 250-ml. culture flasks, inoculated with a spore suspension, and incubated for 7 days at 28° to 30° C. Crude enzyme was then extracted with 25 ml. of distilled water for 3 hours and filtered through cheesecloth. A portion was assayed directly; and a second portion was precipitated with 70% saturated $(NH_4)_2SO_4$, redissolved in the original volume, and assayed.

Although enzymes from this particular culture of *T. viride* showed no ability to improve the extractability of coconut protein, a commercial preparation from *T. viride* grown on wheat bran and sawdust was active

When the residue from enzyme extraction was re-used as a culture medium, succeeding cultures showed increasing activity in terms of their facilitating extraction of protein from coconut flour (Table II).

Twenty grams of coconut flour plus 40 ml. of trace mineral solution were sterilized in 1-liter flasks, inoculated with spore suspensions, and incubated for 8 days at 28° C. Enzymes were then extracted into 100 ml. of distilled water and filtered through cheesecloth. The residue was then re-used to grow succeeding cultures. To assess enzyme activity, 2.0 grams of coconut flour, 20 ml. of water, and 10 ml. of enzyme were incubated for 3 hours, minced in a Waring Blendor for 5 minutes, and filtered through four layers of cheesecloth. The protein in the filtrate was then determined by micro-Kjeldahl analysis (*10*) using a factor of 6.25 to convert N to crude protein.

The composition of the medium on which the fungus is grown has a marked effect on enzyme yield. A variety of media were assessed for their capacity to produce suitable enzymes when 20-gram portions were moistened with the mineral salt solution of Reese and Mandels (*15*). After growth of the fungus, the enzyme was extracted and tested as in the experiments of Table II. The results in terms of the percentage of coconut flour protein extracted were: nonenzymic control, 57; Solka Floc, 57; coconut flour, 64; coconut flour–Solka Floc (1:1), 77, and coconut flour–Solka Floc (2:1), 82. The coconut flour–Solka Floc mixture in a 2 to 1 weight ratio was used in all subsequent work.

Table II. Increased Enzyme Activity of Filtrates from a Series of Cultures Grown on Same Batch of Coconut Flour[a]

	Protein Extraction from Coconut Flour, %		
Serial Culture	Without enzyme	*C. globosum*	*P. westerdijkii*
First	62	63	63
Second	63	64	65
Third	63	67	69
Fourth	63	...[a]	70
Fifth	...	...	84

[a] Growth failure of *C. globosum* or absence of a control analysis.

In determining the effect of growth temperature on enzyme yield, the range between 28° and 40° C. was studied. Growth with sporulation in 5 to 7 days occurred at 28°, 31°, and 32° C. The cultures in this experiment were inoculated into 50 grams of sterile coconut flour–Solka Floc mixture (2 to 1) moistened with 70 ml. of tap water. After 8 days of incubation the enzyme was extracted with 5 volumes of water, precipitated with 80% saturation $(NH_4)_2SO_4$, and dialyzed by gel filtration. To determine relative potencies, 1.0 gram of coconut flour was incubated with 30 ml. of enzyme and saturating toluene at pH 6.0 for 72 hours. After filtering through four layers of cheesecloth micro-Kjeldahl and dry matter determinations were made on the filtrates. At 28°, 31°, and 32° C. the corresponding protein extractions were 55, 67, and 65% (no enzyme control, 43%), and the corresponding dry matter extractions were 23, 40, and 37% (no enzyme control, 17%).

In establishing the effect of the age of cultures on their enzyme yields the coconut flour–Solka Floc medium described above was used and incubation was at 31° C. Enzyme was then extracted and evaluated as described in the preceding paragraph. After 5, 7, 9, and 11 days' incubation the corresponding protein extractions were 69, 74, 80, and 88% (no enzyme control, 62%), and the

Table I. Cellulase Production by Fungi[b]

	C_1, Units/Flask[a]		C_x, Units/Flask[b]	
Organism	Crude	$(NH_4)_2SO_4$ ppt.	Crude	$(NH_4)_2SO_4$ ppt.
Aspergillus terreus	5	5	1180	600
Basidiomycetes sp.	23	28	1750	1520
Chaetomium globosum	38	38	1010	900
Myrothecium verrucaria	23	5	1700	1180
Pestalotiopsis westerdijkii	32	14	2260	2840
Trichoderma viride	14	14	0	0

[a] C_1. Cellulase activity measured using hydrocellulose as substrate (*5*).
[b] C_x. β-Glucanase activity measured using carboxymethylcellulose as substrate (*7*).

corresponding dry matter extractions were 26, 28, 59, and 47% (no enzyme control, 9%). A small improvement of activity in facilitating protein extraction was observed when cysteine was added during treatment with the enzyme. The stimulation was only about 7%, however, at the optimum cysteine concentration (600 μg. of cysteine hydrochloride per ml.) and the observation was not studied further.

Data describing the pH and temperature optima appear in Tables III and IV, respectively. Protein solubilization was not proportional to the enzyme concentration under conditions typical of those used here (Table V).

The reactions leading to improved protein extractability are not at all linear in kinetic plots. The solubilization of dry matter appears to follow classical Schutz kinetics (*16*) (Figure 1), which is characteristic of cellulases acting on insoluble substrates (*11*). In contrast, the extraction of protein deviates markedly from Schutz rule and only approximates first-order kinetics.

These enzymes, when incubated with hydrocellulose (140 units of C_1 per 15 mg. of hydrocellulose) at pH 6.0 and 40° C. in the presence of excess toluene for 72 hours, accomplished a 77% solubilization of the substrate. Determination of the degree of polymerization (DP) of the soluble products (*19*) indicated an average DP of 11. Analysis of the smaller members of the oligosaccharide series of cellulose (*4*) showed that glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose accounted for 32.7, 3.3, 1.8, 1.4, 2.5, and 2.0% of the soluble sugars, respectively.

When the soluble sugars in nonenzymatic and enzymatic extracts of coconut flour were determined, arabinose and glucose were evidently the major sugars released during enzyme treatment along with lesser amounts of galactose (Table VI). One hundred milligrams of dried extract were dissolved in 1.0 ml. of water and eight replicate spots of 0.01 ml. were developed in the pyridine–butanol–water solvent. Spots were then located by spraying with periodate–benzidine, and the corresponding areas of the other chromatograms were extracted into 5 ml. of water. Control sections of paper containing no carbohydrate were also extracted. One-milliliter portions of the extracts were then analyzed for reducing sugars.

In larger scale experiments to produce enough of the various extracts and residues for evaluation of protein quality in the rat, lower extractions were generally seen because of the reduced efficiency of the larger Waring Blendor that was used (Table III). Material balances are shown in Table XI.

Each 100 grams of coconut flour was suspended in 800 ml. of distilled water and held at 40° C. for 3 hours. The slurry was then blended in a large Waring Blendor for 4 minutes and filtered through four layers of cheesecloth. The residue was then resuspended in 500 ml. of water or 400 ml. of enzyme (8 units of C_1 and 2.30 mg. of

Table III. Effect of pH on Enzyme Activity[a] as Measured by Increased Protein Extraction

Enzyme Treatment, pH	Protein Extraction, %	Enzyme Treatment, pH	Protein Extraction, %
3.0	43	6.2	71
4.0	54	6.4	71
5.0	69	7.0	66
5.6	72	3.0–4.0 (no enzyme)	41
5.8	72	5.0–7.0 (no enzyme)	66
6.0	72		

[a] Two grams of coconut flour, 20 ml. of crude enzyme, and 20 ml. of distilled water adjusted to desired pH values and incubated at 40° C. for 3 hours. After filtration through four layers of cheesecloth, Kjeldahl analyses were run on extracts.

Table IV. Temperature Effects on Enzyme Activity[a]

Reaction Temp. ° C.	Protein Extraction, %	Reaction Temp. ° C.	Protein Extraction, %
28	73	50	72
30	82	60	68
40	86	Control (no enzyme)	66

[a] Two grams of coconut flour plus 40 ml. of crude enzyme incubated at temperatures indicated for 3 hours and protein extracted measured as in Table II.

Table V. Protein Solubilization from Coconut Flour in Response to Graded Concentrations of Enzyme[a]

Volume of Enzyme, Ml.	Protein, Extraction, %
0	72
10	78
20	82
30	84
40	85

[a] Two grams of coconut flour incubated at 40° C. in a total volume of 40 ml. with volumes of enzyme indicated for 6 hours. Protein extraction determined as in Table II.

Table VI. Water-Soluble Sugars in Coconut Extracts Prepared with and without Enzyme Treatment

Sugar	Non-enzymatic Extract, Mg./G.	Extract Using Boiled Enzyme, Mg./G.	Enzymatic Extract, Mg./G.
Arabinose	3.7	3.3	30.0
Glucose	13.9	13.8	36.6
Galactose	11.3	12.2	17.2
Disaccharides	1.3	1.3	1.3

Table VII. Extraction of Protein from 1.2 Kg. of Coconut Flour with and without Enzyme Treatment

Process	Extraction of Protein, %	Extraction of Dry Matter, %
No enzyme	49	60
Boiled enzyme	55	60
Enzyme	85	79

protein per ml.) plus 100 ml. of water, adjusted to pH 6.0, and held at 40° C. for 3 hours. After blending for 2 minutes, the contents were filtered as before. A second extraction with 200 ml. of water was then carried out for 30 minutes. The extracts were pooled, and both extracts and residues were lyophilized.

The amino acid composition of the various fractions in grams per 100 grams of crude protein is shown in Table VIII and in terms of the per cent of the rat's requirement in Table IX.

In assessing the nutritional quality of the protein in the various fractions, the NAS-NRC procedure of Campbell (*2*) was basically followed, except that vitamins A, D, and E were provided as percomorph oil and α-tocopherol. The dietary levels in international units per 100 grams of diet were 208, 90, and 6.6 for vitamins A, D, and E, respectively. Because of delayed acceptance of the diets growth performance was poor during the initial week, so PER values in Table X were based on performance between the 8th and 15th days.

Nitrogen balance experiments were carried out to determine crude digestibility of the enzymatic and nonenzymatic extracts and residues to clarify the very poor PER values of the residues—values much lower than their amino acid patterns (Tables VIII and IX) would predict. After 6-day periods the values in Table X were obtained.

Discussion

Tables VII and XI show clearly that using relatively crude enzyme preparations, the extractability of protein from coconut flour can be raised from 49 to 85%. The concurrent removal of about 50% of the crude fiber (Table XI) suggests further that enzymatic treatment may well yield a modified coconut flour of low enough fiber content to be usable as an infant food without the costly operations of extracting protein and producing protein isolate. In either case, the technical feasibility of enzymatically improving the nutritive value of coconut flour is established. Economic considerations can now be investigated.

The very limited screening of fungi for their ability to produce satisfactory enzymes (Table I) indicates that considerable attention needs to be given to selection of the enzyme source. With a broader survey of potentially useful species of fungi and then strain selection within the best species, much more potent enzyme preparations can be anticipated.

Similarly a detailed study of the effect of growth conditions is warranted. Among the critical factors

Table IX. Amino Acid Composition Expressed as Per Cent of Rat's Growth Requirement as Estimated (*14*)

	Per Cent of Requirement[a]			
	Extract		Residue	
Amino Acid	Without enzyme	With enzyme	Without enzyme	With enzyme
Histidine	84	80	80	88
Lysine	50	50	52	52
Isoleucine	67	73	95	80
Valine	98	104	135	113
Leucine	96	100	129	110
Sulfur amino acids	60	56	46	40
Threonine	58	74	86	86
Aromatic amino acids	100	103	125	106

[a] Based on 10% true protein in diet.

Table VIII. Amino Acid Composition of Coconut Flour Fractions Prepared with and without Enzyme Treatment[a]

	Process					
	Nonenzymatic		Boiled Enzyme		Enzymatic	
Amino Acid	Extract	Residue	Extract	Residue	Extract	Residue
Lysine	4.05	3.44	4.31	4.11	4.06	4.28
Histidine	1.92	1.47	1.64	1.81	1.78	2.01
Ammonia	1.64	1.60	1.76	1.67	2.04	2.05
Arginine	14.07	9.68	12.76	12.19	12.69	11.19
Aspartic acid	7.66	8.49	7.60	8.57	7.89	9.45
Threonine	2.64	3.17	3.01	3.24	3.30	3.90
Serine	3.80	4.42	2.36	3.93	3.93	4.60
Glutamic acid	18.38	4.64	16.99	3.80	17.27	14.78
Proline	3.21	3.30	3.23	3.35	3.47	3.79
Glycine	4.26	4.29	4.21	4.37	4.50	4.85
Alanine	3.53	3.59	3.34	3.62	3.75	3.92
Half cystine	1.72	0.86	1.88	0.82	1.43	1.23
Valine	4.80	5.34	4.75	4.28	5.13	5.64
Methionine	1.04	0.88	0.83	1.01	1.07	0.72
Isoleucine	3.32	3.78	3.46	3.88	3.63	3.99
Leucine	5.98	6.52	5.97	6.83	6.29	6.98
Tyrosine	2.48	2.22	2.29	2.23	2.50	2.61
Phenylalanine	3.90	4.38	3.76	4.41	4.17	4.43
Total	88.40	72.07	84.15	72.12	88.90	90.42

[a] Grams of amino acid per 100 grams of crude protein.

Table X. Nutritive Value of Extracts and Residues

Protein Source	Weight Change, G./10 Days	PER	Crude Digestibility of Protein, %
Extract			
Without enzyme	17.5	2.27	83
With enzyme	10.3	1.81 (n.s.)[a]	76 (n.s.)[a]
Residue			
Without enzyme	−1.8	---	62[b]
With enzyme	−5.2	---	44[b]
With boiled enzyme	−2.2	---	---
Casein	26.6	3.18	---

[a] n.s., not significantly different from extract without enzyme at $P = 0.05$ in chi square and simple t test.
[b] Highly significant ($p < 0.01$) compared to same value in same statistical analyses. --- Not determined.

Table XI. Material Balances on 1200-Gram Lots of Coconut Flour

Process	Dry Matter, G.		Protein, G.		Crude Fiber, G.	
	Extract	Residue	Extract	Residue	Extract	Residue
No enzyme	486	596	139	118	9	169
Boiled enzyme	482	598	153	115	13	187
Enzyme	835	250	237	26	19	100

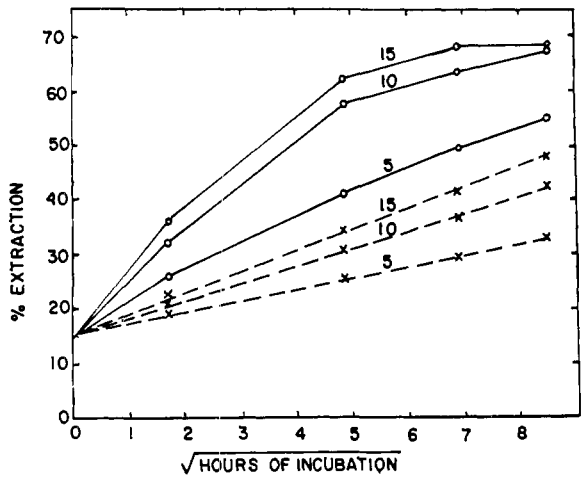

Figure 1. Kinetics of extraction of protein and dry matter

——— Protein extraction
– – – – Dry matter extraction
One gram of residue from aqueous extraction of coconut flour plus the volumes of enzyme indicated were adjusted to pH 6.0, diluted to 50 ml., and incubated at 40°C. in the presence of saturating toluene for 3, 24, 48, or 72 hours. After filtration micro-Kjeldahl and dry matter determinations were made on the filtrates.

needing more precise consideration are the composition of the medium and the number of successive cultures that have been grown on the same support (Table II).

Assuming the use of *Pestalotiopsis westerdijkii* and the coconut flour–Solka Floc medium, the approximate optima for other parameters of the culture are 31–2° C. incubation temperatures and 10- to 11-day incubation periods.

In handling enzymes at their present stage of development, the $(NH_4)_2SO_4$ or some similar precipitation step is a practical necessity in order to concentrate the enzymes. The gel-filtration step then becomes desirable in order to remove excess $(NH_4)_2SO_4$ and to complete the removal of low molecular weight toxic by-products of the fungus growth.

For the enzymatic treatment the pH optimum is 5.6 to 6.0 (Table III). In 3 hours' reaction periods, the temperature optimum lies near 40° C. (Table IV).

A number of questions remain unanswered about the process. It is not at all clear whether the hemicellulase and cellulase activities which have been demonstrated are directly involved in the improved protein extractability. The fact that protein release and dry matter solubilization follow distinctly different kinetic patterns (Figure 1) suggests that the relationship is at most indirect. On the other hand, the hemicellulase and cellulase activities must be directly responsible for the crude fiber reduction.

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