ENZYMIC EXTRACTION OF PROTEIN FROM COCONUT FLOUR (*Cocos nucifera*)

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PLATES

I. STATEMENT OF THE PROBLEM

Coconut flour protein is of greater nutritional value than that of coconut protein isolate, but the high fiber of the flour is of concern in infant and child feeding (29, 30). There are many reports to indicate that the cellulase types of enzyme are of immense help in the degradation of various cellulosic substrates including raw and cooked vegetables. It may be possible to reduce the fiber content to a satisfactory level by suitable enzymic treatment or to strip out the fiber prior to protein extraction. This latter process might increase the recovery of the flour protein isolate. Since the biological value of the flour protein exceeds that of the isolate (30), improving the efficiency of the extraction would be expected to improve the nutritive value of the isolate.

In this dissertation attempts were made to select a suitable mold for the preparation of a potent, freely diffusing cellulase type of enzyme which could fulfill the above expectations.

II. INTRODUCTION AND LITERATURE REVIEW

At present at least one third of the world's population is malnourished or underfed. The dietary surveys conducted so far by many individuals and groups have shown that the present diet of a great proportion of the human race is deficient in protein. This is particularly true of the people of the emerging nations where chronic malnutrition is found even among families with large livestock holdings. The main dietary source of the people in underdeveloped countries is of plant origin, and especially the post-weaned children receive mostly cereal grains and tuber flours. This and restricted intake of animal protein due to its cost and availability create the problem of inadequacy in protein intake and amino acid balances; however, careful planning to use local plant foods in proper combination and proportion, should help to eliminate the protein deficiency for the most part, though not completely. Developments of such foods as INCAPARINA are already under way in Latin American countries and in Haiti.

An important group of the plant kingdom worth considering as a source of protein supplement in day to day diets is the "Oil Seeds." Soybean, cotton seed, peanut, sesame and coconut are of major interest in the above group.

Coconut is a cash crop in many countries, and the copra or the dried coconut has been an article of international commerce for a long time. Table 1 (3) shows world production data of copra. Usually

Table 1

World Production of Copra* (1963)

Country	Production in 1,000 metric tons
Asia	
Ceylon	240.4
States of Malaya	139.0
India	259.0
Indonesia	403.5
North Borneo	33.0
Philippines	1,511.3
Thailand	20.8
Viet Nam	25.5
Oceania	
Br. Solomon Island	25.6
Fiji	41.9
French Polynesia	24.9
New Guinea	93.3
Latin America	
Mexico	203.9
West Indies	35.0
Africa	
Mozambique	62.0

*Countries producing 20,000 or more metric tons of copra.

the coconut is dried in the sun and often stored under damp conditions till shipment. The humidity and other conditions prevailing at storage favor the growth of microorganisms which results in the production of toxic substances and off flavors and odors thus rendering the copra unfit for human consumption. So it is essential to eliminate the microbial contamination in order to use the copra and/or copra meal as a protein supplement in the human diet.

Since currently, the main objective of processing copra is to get maximum extraction of oil, the protein fraction has been largely neglected, and the meal left over after the oil extraction is generally used as manure or livestock feed. The oil is extracted by either one of the following two methods.

1. The expeller process where high temperature and pressure physically squeeze the oil out and,
2. Solvent extraction method where the oil is removed by an organic solvent such as hexane.

More often the protein in the press cake is heat-damaged and its biological value is reduced in the former case, while the problem of residual solvent is detrimental in the latter method.

However, specially prepared expeller coconut meal (cake) contains 20 to 25 percent protein and about 10 to 12 percent fiber which limits its use as a supplement in infant and child feeding (29). So it became a "must" either to reduce or totally eliminate the fiber content of the meal by various possible means before testing the nutritional value of the protein in the coconut meal. Because albumins and globulins

are the major proteins of the oil seeds, these can be extracted by peptization with water leaving behind part of the protein and most of the fiber. There are reports in the literature to show that the nutritive value of coconut flour is greater than that of coconut protein isolate (30). According to Van Veen (30) the fiber can be reduced by fermentation but only at considerable risk of bacterial contamination. So it appears probable that an enzyme capable of reducing the fiber of coconut flour to a satisfactory level within a few hours could be of immense help. This enzymic treatment of the flour to strip out the fiber prior to protein extraction might improve the efficiency of extraction thereby improving the nutritive value of the isolate.

The following are the various methods available to date for coconut processing.

1. The Lava Process (31) was developed by Lava in 1937 to extract the oil from fresh shredded coconut meat by water extraction and subsequent separation of oil from protein. The by-products are the press cake with 8 percent protein, dried protein concentrate containing 75 percent protein in fairly pure state and an aqueous sugar-mineral solution. Dried protein concentrate could be used to replace casein in human and animal feeding.

2. Robledano-Luzuriage Process, 1948 (31). This is a "wet process" in which the meat from the ripe nut is ground and pressed to obtain an emulsion of oil and water extractions which by subsequent treatments separated into oil and water phase. Heating the water phase to 200°F yields the protein concentrate containing around 60 percent protein. The residual meal is reported to be suitable for

direct human consumption.

3. Carver-Greenfield Process (31). The essential feature of this process is the evaporation of the water in the ground meal at low temperature in the presence of excess of oil followed by pressing the slurry to separate oil from non-fat constituents.

4. Hiller's Process, 1958. In this process, the kernel is removed from the nut after cooking the mature nut. The sliced kernel is then dried rapidly and the oil is extracted with an expeller. The cake is ground into a meal and is of good quality. The processing time is around three hours.

5. Azeotropic Extraction, 1963 (31). This process was developed by Central Food Technological Research Institute (CFTRI) in which the kernel after separation from the shell is milled and mixed with solvent heptane. The extraction is carried out until the material is free from moisture and oil. Then the meal is passed through a micro-atomizer and powdered. It can be used as such in edible formulations. Eighty per cent of its protein is water extractable.

6. Kraus-Maffei (KM)/CFTRI Process. In this process the kernel obtained from steamed nut is pressed in a screw press. The "milk", free from fibrous meal, is separated into oil phase and water phase by centrifugation. The water phase is concentrated to get "coconut honey" rich in heat or acid coagulable protein.

In the modified CFTRI/KM process where steaming of the nut is abandoned, the recoveries of oil and protein are improved.

7. In 1960 Peters (32) presented a lab-scale "wet extraction" technique which comprises conventional methods of grinding, aqueous extraction, pressing and isoelectric precipitation. Then it was augmented by employing saline and alkaline solutions to increase protein yield. The limitation of this process is that the oil recovery is low compared to that of copra pressing.

As early as 1919 Johns, Finks and Paul (16) reported that coconut globulin produced a normal growth response in rats fed as a sole source of protein while coconut press cake produced an almost normal growth response. Since then many workers have tried to compare the nutritive value of coconut meal and corn in terms of Protein Efficiency Ratio (PER), Biological Value, and Digestibility in ad libitum feeding experiments. Some observed high Biological Value and digestibility for coconut meal (23, 36, 24) while others found no difference between the two (25).

Even though Jones, Finks and Johns (18) could not find any difference in Biological Value between solvent extracted and screw pressed meal, it is widely accepted that the former is generally more nutritive than the latter. According to Mitchell et al. (24) the coconut meal produced by an extraction process in which the temperature never exceeded 75°C had a higher biological value than a product prepared by the "usual drastic methods." When the meal was subjected to various degrees of heat treatment and the nutritive value of the resulting products was evaluated, the results showed that the nutritive value as well as available lysine and HCl-soluble protein decreased as the processing temperature increased (4).

The PER of expeller pressed meal has been variously reported from 1.2 to 2.3 (31). The wide variations experienced are evidently due to different degrees of protein damage caused by the high heating encountered in pressing. Tryptophan, lysine, and methionine are the limiting amino acids of which lysine is the most limiting one (4, 31). The low nutritive value of commercially produced coconut meal has been attributed to the possible inactivation of lysine during processing (8, 32). Coconut meal as well as coconut protein isolate showed a remarkable supplementary value to poor cereal and tuber flour diets (19, 35, 32, 39).

The use of coconut oil meal in diets for poultry and other animals has been reported by various workers. The results with poultry have been so poor that several reports suggested the presence of some toxic or growth suppressing factor. Chick studies have usually resulted in poor growth, poor efficiency of feed utilization and high mortality. Laying hens lost weight and productivity (10, 21, 9). However, recently Thomas and Scott (41) demonstrated that copra meal does not contain any toxic or growth depressing activity when properly supplemented and balanced in terms of all nutrients. Their report also confirmed the earlier reports (8, 32) that lysine is more severely damaged in expeller meals than in solvent processed meals.

Now-a-days, supplementation of diets with commercial cellulase type enzyme preparations is becoming more and more common. Use of fungal and bacterial enzymes to supplement the barley rations for

chicks showed significant improvement in growth rate and feed efficiency (14, 47). The studies on the effect of enzyme treated barley on the performance of breeding hens resulted not only in the increased feed efficiency but also increased egg production and hatchability (27). According to Toyama (44), Morimoto in a Japanese patent claims a 35 percent increase in egg production as a result of adding crude Trichoderma viride cellulase to barley-based layer rations.

III. MATERIALS AMD METHODS

A. Microbiological Procedures

1. Screening of Cellulolytic Fungi. (Table 2)

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, were compared for their ability to elaborate freely diffusing, extracellular cellulolytic enzymes when grown on coconut flour and trace mineral solution of Reese and Mandels (34). The crude enzyme was prepared following the procedure of Toyama (42).

2. Cultural Medium

(i) Serial Culture Experiment (Table 3)

P. westerdijkii and *C. globosum* were grown on coconut flour and trace mineral solution for eight days, and the enzyme was extracted. The residue containing the coconut flour and the fungal mycelium was dried, ground, and used again to grow the molds. This procedure was repeated until the molds were unable to grow any more on that particular medium.

(ii) Modification of the Cultural Medium (Table 4)

In order to select the medium best suited to support growth and active enzyme production by *P. westerdijkii* several media were prepared using coconut flour and solka-floc in various proportions, and the fungus was grown on them for eight days. The enzyme produced was extracted and tested for its capacity to facilitate the extraction of protein from coconut flour.

3. Extraction and Partial Purification of the Extracellular Enzyme (Fig. 1, 2 and Table 5)

The crude enzyme was extracted from flask or tray cultures. The enzyme precipitated between 30 and 80 percent saturation of $(NH_4)_2SO_4$ was collected by refrigerated centrifugation at 2500 x G for 30 minutes and redissolved to one-tenth of the original volume with distilled water. The concentrated enzyme was filtered through a glass fiber filter, and one ml was put on the Sephadex G-25 column (1.5 x 15.0 cm) and eluted with distilled water. One ml fractions were collected and spot tests were made using 0.05 ml of eluate and 0.4 ml of p-nitrophenyl-β-D-glucopyranoside. After 5 minutes 0.1 ml of 25 percent K_2HPO_4 was added, and the appearance of the yellow color caused by p-nitrophenol was considered as an indication of enzyme activity (12). This procedure was repeated with a larger column to collect the active portion of the enzyme in large scale preparations.

The possibility of loss in activity of the enzyme during gel filtration was also considered but were not serious.

4. Effect of Changes in Cultural Conditions on the Enzyme Production

(i) Optimum Temperature (Table 6)

The enzymes produced at different temperatures by P. westerdijkii were compared for their ability to implement the extraction of protein from coconut flour.

(ii) Age of Culture (Table 7)

Cultures were harvested at different intervals of time, and the enzyme preparations were compared for their capacity to increase protein extractability from the coconut flour.

B. Assay Procedures

1. C_1 Assay.

This assay using hydrocellulose as substrate was performed according to the procedure of Flora (11). The decrease in turbidity by enzymatic action was measured in a Klett-Summerson colorimeter using the red filter.

2. Carboxymethyl Cellulase Assay (CMC-ase assay)

This assay was performed following the procedure of Hash and King (13) where the reducing groups produced by the enzymatic action on carboxymethyl cellulose (CMC-70-L, Hercules Powder Co.) were measured by the Nelson-Somogyi procedure (26, 38).

3. Aryl β-Glucosidase Assay

This assay using p-nitrophenyl- β-D-glucopyranoside as substrate was performed according to the procedure of Hash and King (12).

4. Activation of Enzyme by Cysteine Hydrochloride (Table 8)

The optimum level of cysteine hydrochloride required to activate the P. westerdijkii enzyme was studied using different quantities of cysteine hydrochloride in the reaction mixture (46).

5. Studies on Protein Extraction by P. westerdijkii Enzyme

(a) Bench Scale:

(i) Optimum Temperature (Table 9 and Fig. 3)

This was studied by incubating the coconut flour and the enzyme mixture for three hours at different temperatures ranging from 28 to 60°C.

(ii) Optimum pH: (Table 10 and Fig. 4). The pH at which the enzyme could cause maximum protein extractability from coconut flour was studied by varying the pH's of the reaction mixtures from 3.0 to 7.0.

(iii) Effect of Concentration of the Enzyme on the Protein Extraction (Table 11). A comparative study was made using different volumes of crude enzyme to extract the protein from the coconut flour.

(iv) Effect of Incubation Time (Table 12, and Fig. 5 and 6). The variation in protein and dry matter extraction caused by pretreatment with enzyme was determined by incubating 5, 10, and 15 ml of the enzyme with one gram of the coconut flour residue for 3, 24, 48 and 72 hours.

(b) Processing of Coconut flour for Rat Feeding Experiments: (Tables 13 and 14)

The protein from each 100 gm of coconut flour was extracted with 1500 ml of distilled water or distilled water plus partially purified enzyme. The protein and the residue preparations were lyophilized in trays and used for preparing the diets.

C. Chemical Analysis

1. Timell Analysis: The hydrolytic products formed from hydrocellulose by the P. westerdijkii enzymatic action and the average

degree of polymerization (D.P.) of the products, were determined according to the procedure of Timell (40).

2. Reducing Sugar Analysis: Following the procedure of Nelson and Somogyi (26, 38) the amount of water soluble reducing sugars present in various coconut milk preparations was estimated.

3. Paper Chromatography (Table 22). To identify the various sugar fractions contained in the coconut milk preparations, paper chromatograms were developed on Whatman No. 1 filter papers for 20 hours either in the ethylacetate-pyridine-water solvent of Jermyn and Isherwood (15) or in the pyridine-n-butanol-water solvent and sprayed with the periodate-benzidine spray of Cifonelli and Smith (6).

4. Nitrogen Determination by Microkjeldahl Procedure: One ml or less of sample was heated with one ml of digestion mixture for 12 hours in a digestion oven at about 300°C in pyrex test tubes. After cooling the tubes, 3 ml of water, 3 ml of 3.3N NaOH and 2 ml of Nessler's reagent were added, mixed and read in a Spectronic 20 colorimeter after standing for 10 to 15 minutes (17).

5. Proximate Analysis: This was done for moisture, crude protein, ether-extract, crude fiber, and ash according to the procedures described in the A.O.A.C. manual (1).

6. Amino acid Analysis (Tables 15, 16, 17 and 18). Amino acid content of various milk preparations was analyzed in Beckman-Spinco amino acid analyser after hydrolyzing the samples in sealed thick walled glass test tubes for 40 hours (37). Correction was

made for loss of amino acid if any, during hydrolysis, and internal standards of nor-leucine and β-guanidino-alanine were included with the long and short columns respectively.

D. Animal Evaluation

1. Determination of PER (Table 19). Following the procedure of Campbell (2) diets were prepared at 9 and 10 percent levels of protein, using various coconut preparations as protein source. Twenty-one day old weanling male rats of Sprague-Dawley strain weighing 55 ± 10 gm, (mean weight among the groups being 52 ± 1 gm) were kept in individual wire-screen cages. Food and water were given ad libitum. The experiments lasted 7 and 10 days for coconut residue and milk preparations respectively. The food intake was measured every day.

2. Determination of Apparent Digestibility (Table 20)

Sprague-Dawley strain male rats were individually kept in metabolism cages. Food (containing 9 and 10 percent protein) and water were given ad libitum. The food intake was measured every day. The urine, collected under toluene, and the feces were collected over six days of the experimental period. The nitrogen content of urine and feces was determined by the A.O.A.C. macrokjeldahl method (1).

E. Enzymological Observations

1. Product Analysis (Table 21). The products formed from hydrocellulose by the action of P. westerdijkii enzyme were analyzed according to Cole and King (7), and Timell (40). The average D.P. of the products was also determined.

2. Degradation of Filter Paper and Other Carbohydrates (Plate 1 and Tables 23 and 24).

The ability of the enzyme to degrade pieces of Whatman No. 1 filter paper as well as other carbohydrates was studied (42, 20).

3. Vegetable Processing. (Plates 2 - 11)

Thin slices, pieces or sections of various vegetable products were treated with partially purified P. westerdijkii enzyme. Observations were made after 12 and 48 hours of incubation to establish the extent of degradation of vegetable products.

IV. RESULTS AND DISCUSSION

1. Screening of Cellulolytic Fungi.

Six cellulolytic molds, A. terreus - Q.M.72f, Basidiomycetes sp. - Q.M. 806, C. globosum - Q.M. 459, M. verrucaria - Q.M. 460, P. westerdijkii - Q.M. 381, and T. viride - Q.M. 6a, were tested for their capacity to produce freely diffusing, extracellular enzymes to hydrolyse crystalline and amorphous cellulose by growing them on the medium of coconut flour and trace mineral solution (34). The assays for total C_1 and CMC-ase activity (11, 13) produced by the above molds in each flask were compared as seen in Table 2. The wide variations ranging from 5 to 38 and from 0 to 22,600 units of C_1 and CMC-ase per flask respectively, are quite obvious from the table. Concentration of the crude enzyme preparations by precipitation with $(NH_4)_2SO_4$ at 70 percent saturation resulted in appreciable loss in activity in many cases. Since C. globosum and P. westerdijkii showed high C_1 and CMC-ase activities respectively, both were chosen for further studies.

T. viride, a potently cellulolytic fungus, was not able to produce active enzyme for improvement of the extractability of protein from coconut flour when grown on the medium of coconut flour and trace mineral solution. However, a commercial crude enzyme preparation showed a marked improvement in protein extraction when used to treat coconut flour.

2. Serial Culture Experiment.

C. globosum and P. westerdijkii were grown on the same

coconut flour plus trace mineral substrate repeatedly, and the enzyme preparations obtained were compared for their potency. The results are shown in Table 3.

The gradual improvement in the extractability of the protein from the coconut flour by enzymic treatment, shown in the above table suggests that at each step a part of the easily available nutrients ought to have been stripped out of the coconut, thus, forcing the organisms to produce more and more active enzyme to degrade the fibrous portion of the medium in order to get the energy to support growth. C. globosum was unable to grow after the third culture while P. westerdijkii grew until the fifth culture. A 10 percent increase of coconut protein extractability seen in the non-enzymic treatment group at the fifth culture level makes that part of the data not quite reliable. From this stage onwards only P. westerdijkii was considered.

3. Selection of the Cultural Medium Best suited for Active Enzyme Production by P. westerdijkii.

Since the previous experiment suggested that the more restricted was the easily available carbohydrate in the medium, the higher the potency of the enzyme produced by the organism when grown on it, various growth media containing different proportions of the coconut flour and the solka-floc were used for the enzyme preparation. When the potency of the enzymes was tested in terms of the ability to improve the extractability of protein from the coconut flour by the enzymic treatment, the one obtained from the growth medium containing coconut flour and solka-floc in the ratio of 2:1 proved

Table 2

Screening of Cellulolytic Fungi*

Organism	C_1 Activity, Units/Flask		CMC-ase Activity, Units/Flask	
	Crude	70% $(NH_4)_2SO_4$ Concentrate	Crude	70% $(NH_4)_2SO_4$ Concentrate
Aspergillus terreus	5	5	1,180	600
Basidiomycetes sp.	23	28	1,750	1,520
Chaetomium globosum	38	38	1,010	900
Myrothecium verrucaria	23	5	1,700	1,180
Pestalotiopsis westerdijkii	32	14	2,260	2,840
Trichoderma viride	14	14	0	0

*Exactly 5 gm of coconut flour plus 25 ml of the trace mineral solution of Reese and Mandels (34) were placed in 250 ml Erlenmeyer flasks with cotton stoppers and sterilized at 121°C for 30 minutes. The flasks were then inoculated with 2 ml of spore suspensions and incubated at room temperature (28 to 30°C) for seven days after which the crude enzyme was extracted with 25 ml of deionized distilled water for three hours and expressed through cheese cloth. Part of the crude enzyme was concentrated by precipitating at 70 percent saturation of $(NH_4)_2SO_4$ and the precipitate was redissolved in distilled water to the original volume. C_1 and CMC-ase assays were done following the procedures of Flora (11) and Nash and King (13) respectively.

Table 3

Enhancement of Enzyme Activity by Serial Culturing on the Same Substrate by *C. globosum* and *P. westerdijkii**

Serial Culture No.	Protein Extraction (%)		
	Control (no enzyme)	*C. globosum*	*P. westerdijkii*
1	62.3	62.5	63.0
2	63.0	64.3	64.7
3	62.8	67.2	68.6
4	62.7	--	70.2
5	73.1	--	83.6

Symbol - : no growth

*Exactly 20 gm of coconut flour plus 40 ml of the trace mineral solution of Reese and Mandels (34) were placed in one liter Erlenmeyer flasks with cotton stoppers and sterilized at 121°C for 30 minutes. The flasks were inoculated with spore suspensions and incubated at room temperature for eight days, after which the crude enzyme was extracted with 100 ml of distilled water for three hours and expressed through the cheese cloth. The coconut flour along with the fungal mycelium was dried, ground in a Wiley Mill, placed in the flask, moistened with 40 ml of the trace mineral solution, sterilized and reinoculated with spore suspension. This was repeated until the organisms ceased to grow. For determining the potency of the enzyme, 2 gm of coconut flour plus 20 ml of distilled water plus 10 ml of crude enzyme were placed in a 100 ml beaker and incubated at room temperature for three hours; at that time the contents of the beaker were transferred to a Waring blendor, blended for five minutes and filtered through four layers of cheese cloth. The filtrate (milk) was then analyzed for its nitrogen content by the microkjeldahl procedure (17). Using a conversion factor of 6.25, the crude protein content was calculated.

to be the best (Table 4). Very poor growth of the mold on the medium composed of only solka-floc indicates that the energy source in terms of carbohydrate alone cannot support the growth of the organism.

4. Preparative Scale, Extraction and Partial Purification of P. westerdijkii Enzyme.

The organism exhibited the capacity to grow on the coconut flour-solka-floc (2:1) medium plus tap water in place of the trace mineral solution of Reese and Mandels (34). The crude enzyme was extracted with five parts of distilled water (V/W) following the procedure of Toyama (42). Use of the crude enzyme resulted in large corrections for enzyme nitrogen after treatment with the coconut flour. A thick, dark brown, precipitate was obtained between 30 and 80 percent saturation of $(NH_4)_2SO_4$ and redissolved in distilled water. When the concentrated enzyme was passed through the Sephadex G-25 column, two distinct bands were seen. The first one was a fast moving, highly active, dark brown band with a negligible amount of nitrogen, and the second was a slow moving, slightly active, yellow band having almost all the nitrogen of the concentrated enzyme (Fig. 1). Loss of activity was noticed during the gel filtration step; however, the activity pattern of the enzyme remained the same (Table 5 and Fig. 2), and there was a slight increase in the specific activity (0.119 and 0.140 units of C_1 per mgm protein before and after the gel filtration respectively.)

Table 4

Selection of the Cultural Medium Best suited for Active Enzyme Production by *P. westerdijkii**

Cultural Medium	Protein Extraction (%)
Coconut flour	64.0
Solka-floc	57.0
Coconut flour + Solka-floc (1:1)	76.9
Coconut flour + Solka-floc (2:1)	82.0
Control (no enzyme)	57.0

*The mold was grown on 20 gm of sterile medium in 500 ml Erlenmeyer flasks for eight days at room temperature after which the enzyme was prepared as mentioned in Table 3.

Exactly 2 gm of coconut flour plus 40 ml of enzyme were placed in a 100 ml beaker and incubated at room temperature for three hours. The milk was extracted, and its protein content was determined as described in Table 3.

Fig. 1

Preparative Scale Extraction and Partial Purification of *P. westerdijkii* Enzyme*

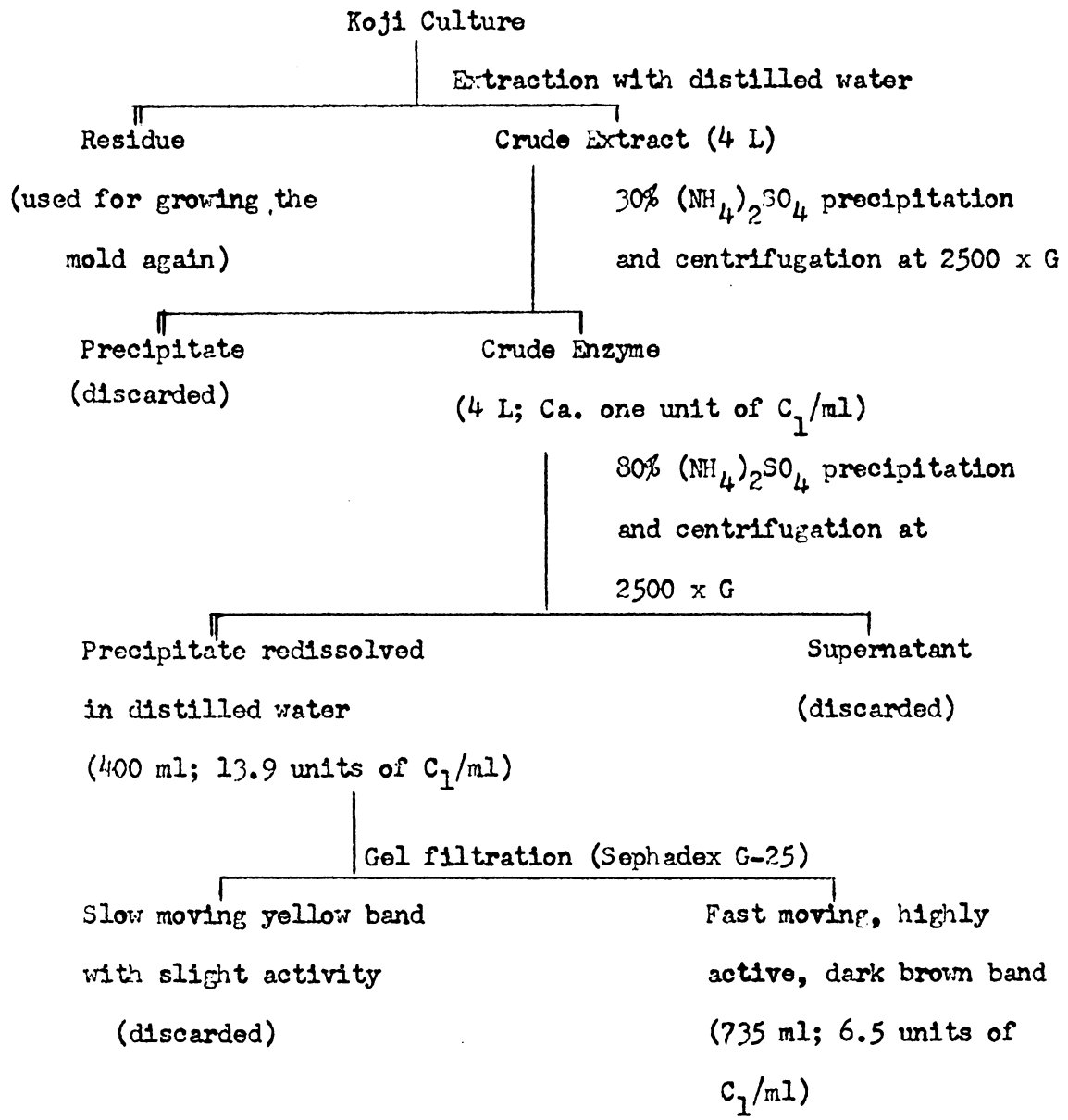


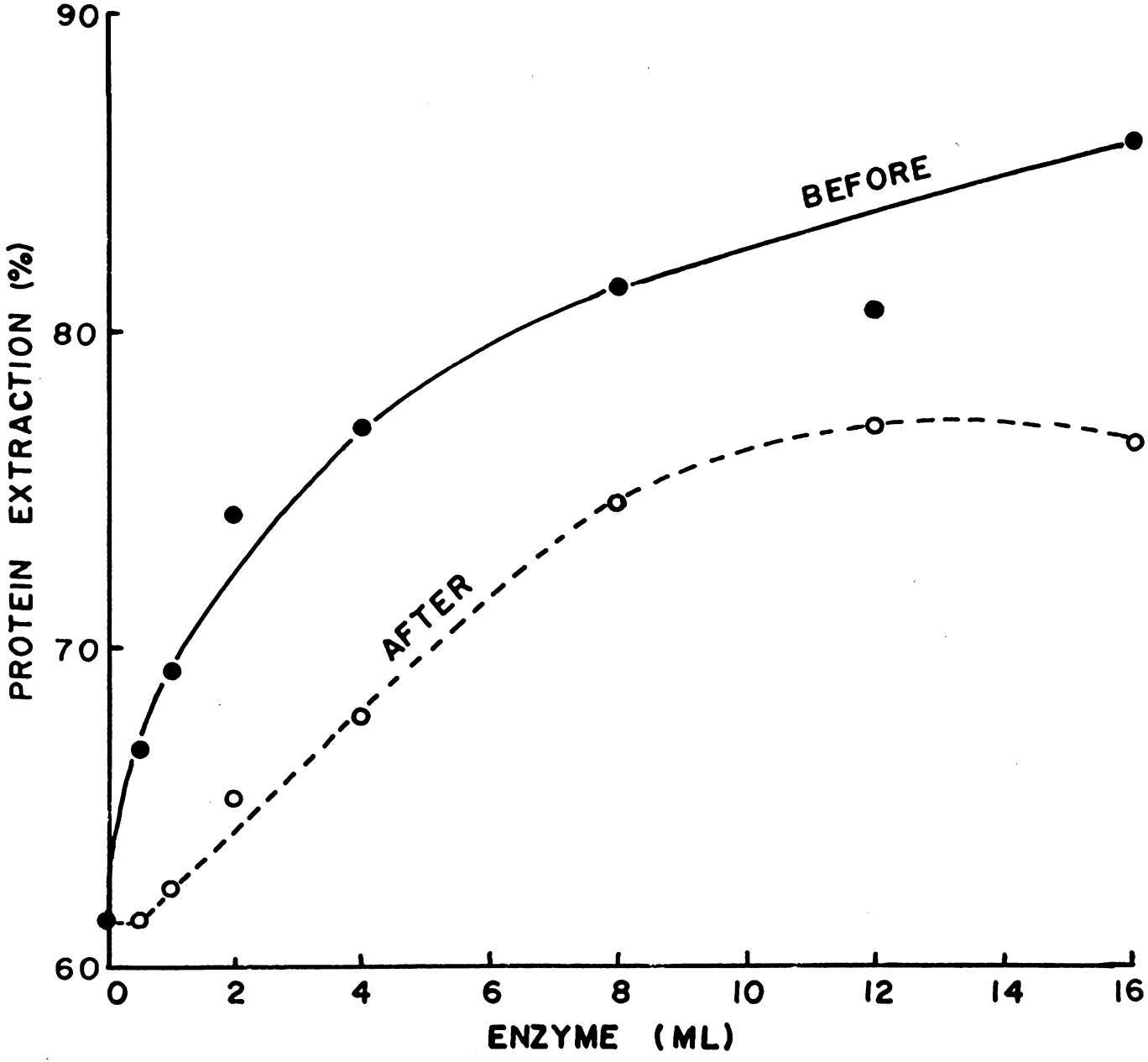
Table 5

Determination of Potency of P. westerdijkii Enzyme before and after Gel filtration*

Volume of Enzyme (ml)	Protein Extraction (%)	
	Before	After
0.0	61.5	
0.5	66.9	61.5
1.0	69.4	62.5
2.0	74.2	65.2
4.0	76.9	67.7
8.0	81.2	74.5
12.0	80.6	76.9
16.0	85.8	76.3

*Two gm of coconut flour plus the specified volume of enzyme were placed in 100 ml beaker and the final volume was made up to 40 ml. A few drops of toluene were added, and the beakers were incubated at 40°C for 72 hours after which the milk was extracted and analyzed for its crude protein content as descibed in Table 3. The volume of the enzyme was almost doubled after gel filtration. Sephadex G-25 was used for this purpose.

Fig. 2. Determination of Potency of P. westerdijkii Enzyme before and after gel filtration


90
80
70
60
PROTEIN EXTRACTION (%)
BEFORE
AFTER
0
2
4
6
8
10
12
14
16
ENZYME (ML)

5. Effect of Changes in Cultural Conditions on the Enzyme Production.

Attempts were made to grow the fungus at different temperatures ranging from 28 to 40°C. Excellent, cottony white, mycelial growth was observed at all temperatures except 40°C, at which temperature the organism did not grow. The cultures sporulated on the fifth, fifth, and the seventh day at 28, 30 and 31°C respectively. The fungus elaborated highly active enzyme at 31°C indicating that it is the optimum temperature (Table 6).

In order to determine the effect of the age of the culture on enzyme production, the mold was grown for up to 11 days at 31°C and harvested at 5, 7, 9 and 11 days for processing. The enzyme preparations were compared for their capacity to increase protein and dry matter extractability from the coconut flour. Table 7 shows that P. westerdijkii is capable of elaborating the greatest amount of active enzyme when grown at 31°C for 11 days as evidenced by the marked increase in protein and dry matter extraction. Whether growth for more than 11 days would further improve the enzyme yield was not studied.

6. Activation of P. westerdijkii Enzyme by Cysteine-Hydrochloride

There are numerous reports to show that many enzymes can be activated by sulfhydryl reagents at optimum levels. An attempt was made using cysteine-hydrochloride at levels ranging from 0 to 0.8 mg/ml of enzyme. A slight improvement in the extractability of protein and dry matter was observed when 0.6 mg of cysteine-hydrochloride was used per ml of enzyme (Table 8). Similar

Table 6

Determination of Optimum Temperature for Enzyme Production by P. westerdijkii*

Growth Temperature (°C)	Protein Extraction (%)	Dry Matter Extraction (%)
28	55.0	22.6
31	66.7	39.9
32	65.0	37.1
40	organism did not grow	
Control (no enzyme)	42.7	16.5

*Fifty gm of coconut flour-solka-floc mixture (2:1) plus 60 to 80 ml of tap water were placed in one liter Erlenmeyer flasks with cotton stoppers and sterilized at 121°C for 30 minutes. The flasks were inoculated with spore suspension and incubated at different temperatures for eight days. Then the crude enzyme was extracted and partially purified as described in Table 3 and Fig. 1.

To determine the potency of enzyme, one gm of coconut flour plus 30 ml of partially purified enzyme adjusted to pH 6.0 were placed in a 100 ml beaker and incubated at 40°C. A few drops of toluene were added to prevent bacterial contamination. After 72 hours the contents of the beaker were emptied into a Waring blender, blended for 5 minutes, and filtered through four layers of cheese cloth. The nitrogen content of the filtrate was determined by the micro-kjeldahl procedure and converted into crude protein using a conversion factor of 6.25.

Table 7

Determination of the Effect of the Age of Culture on Enzyme Production by P. westerdijkii

Age of Culture (Days)	Protein Extraction (%)	Dry Matter Extraction (%)
5	69.2	25.8
7	74.4	28.1
9	79.6	58.6
11	87.6	46.8
Control (no enzyme)	62.4	8.9

*Fifty gm of coconut flour-solka-floc mixture (2:1) plus 60 to 80 ml of tap water were placed in one liter Erlenmeyer flasks with cotton stoppers and sterilized at 121°C for 30 minutes. The flasks were inoculated with spore suspension and incubated at 31°C. The cultures were harvested at different intervals of time. The crude enzyme was prepared and partially purified as shown in Table 3 and Fig. 1.

To test the potency of the enzyme, coconut milk from the coconut flour was prepared and its crude protein content was determined as described in Table 3.

Table 8

Activation of P. westerdijkii Enzyme by Cysteine Hydrochloride*

Cysteine HCl (mg)/ml Enzyme	Protein Extraction (%)	Dry Matter Extraction (%)
Control (no enzyme)	3.0	15.5
0.0	62.3	41.5
0.2	64.0	41.5
0.4	64.0	40.9
0.6	66.0	42.9
0.8	58.3	40.2

*One gm of coconut residue (dry matter left over after protein extraction with water) plus 10 ml of enzyme plus 40 ml of distilled water were placed in a 100 ml beaker with different quantities of cysteine hydrochloride and the pH was adjusted to 6.0. A few drops of toluene were added to prevent bacterial growth. After 72 hours incubation at 40°C, the milk was prepared and the crude protein content was determined as mentioned in Table 3. Correction was made for the nitrogen content of the cysteine hydrochloride added.

results were observed by Verma, et al. (46) in their studies with an enzyme preparation from Curvularia lunata.

7. Studies on Protein Extraction by P. westerdijkii.

The literature survey showed no evidence of any publication dealing with the P. westerdijkii enzyme system. Experiments were designed to study the optimum conditions such as temperature and pH, for the greatest activity of the enzyme. The effects of the concentration of enzyme and of incubation were also studied. The data in Table 9 clearly show that the enzyme causes maximum protein extractability of 86.2 percent from the coconut flour at 40°C. The activity went down sharply corresponding to further increase in the incubation temperature (Fig. 3).

The results of the pH studies are given in Table 10 and Fig. 4. From the table it is obvious that the enzyme was more active at pH 6.0 than at any other pH. The protein extractions at pH 3.0 and 4.0 by the enzymic treatment are comparatively poor which suggests a possible inactivation or denaturation of the enzyme.

The results obtained for an experiment on the enzyme concentration are shown in Table 11. The increase in concentration certainly helped to extract higher percentages of protein from the coconut flour, but use of more than 20 ml of enzyme per gram of flour was of little value.

The effect of various incubation times is shown in Table 12, and Fig. 5 and 6, from which it is evident that the major portions of the protein and dry matter were extracted within

Table 9

Determination of Optimum Temperature for *P. westerdijkii* Enzyme Activity and Protein Extraction*

Incubation Temperature (°C)	Protein Extraction (%)
28	73.0
30	81.5
40	86.2
50	72.0
60	68.0
Control (no enzyme)	66.1

*Exactly 2 gm of coconut flour plus 40 ml of crude enzyme were incubated at various temperatures in 100 ml beakers. After three hours the milk was extracted and the protein content was determined as mentioned in Table 3.

Fig. 3. Determination of Optimum Temperature for P. westerdijkii Enzyme Activity and Protein Extraction

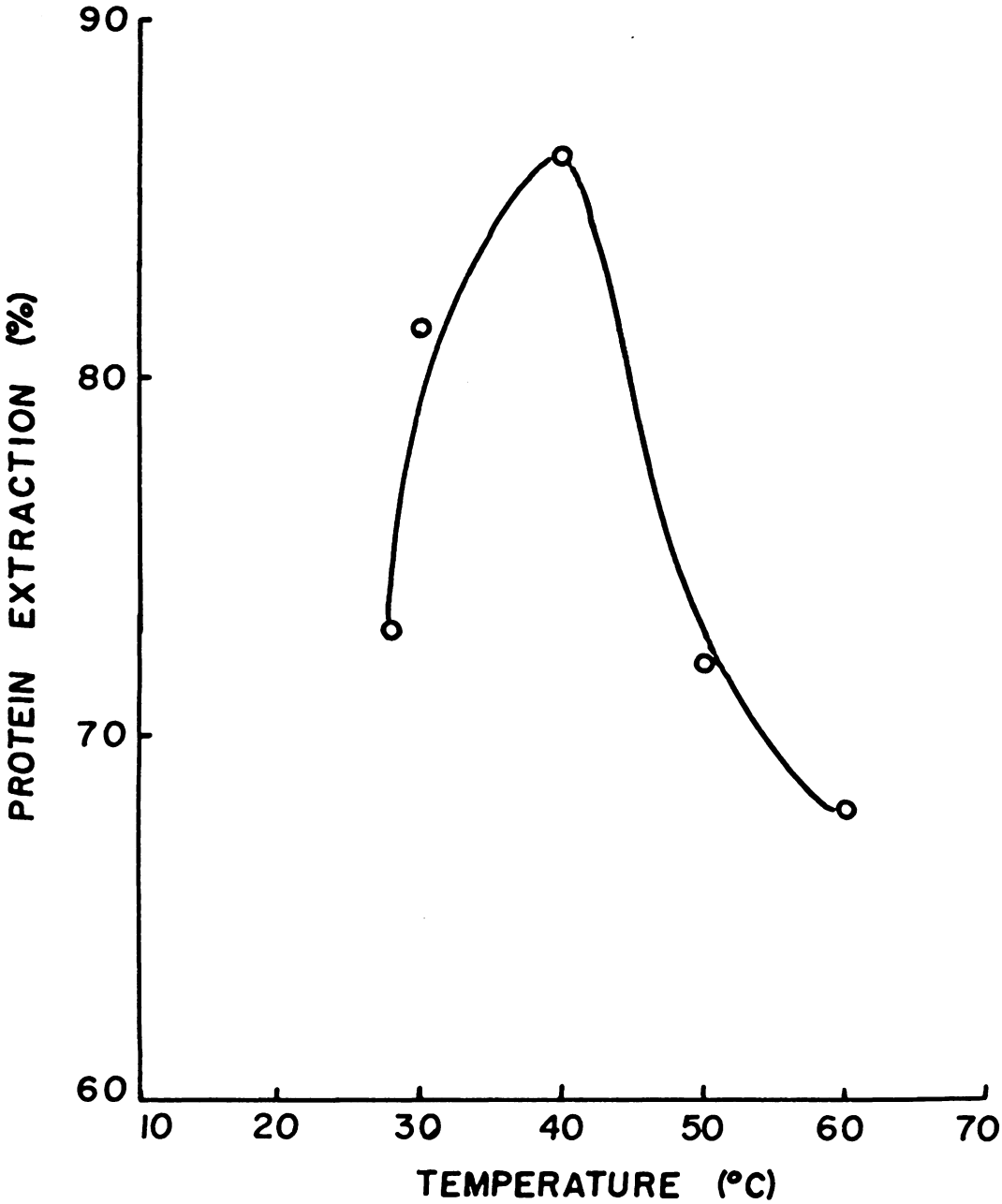

90
80
70
60
PROTEIN EXTRACTION (%)
10
20
30
40
50
60
70
TEMPERATURE (°C)

Table 10

Determination of Optimum pH for *P. westerdijkii* Enzyme Activity and Protein Extraction*

pH	Protein Extraction (%)
3.0	43.0
4.0	53.5
5.0	69.0
5.6	71.8
5.8	72.0
6.0	72.3
6.2	70.7
6.4	71.2
7.0	66.0
Control (no enzyme) for pH 3.0 and 4.0	41.0
Control (no enzyme) for pH 5.0, 6.0, and 7.0	66.0

*Two gm of coconut flour plus 20 ml of crude enzyme adjusted to various pH's plus 20 ml of distilled water were placed in a 100 ml beaker and incubated at 40°C for three hours. The milk was extracted and its protein content was estimated as described in Table 3.

Fig. 4. Determination of Optimum pH for P. westerdijkii Enzyme Activity and Protein Extraction

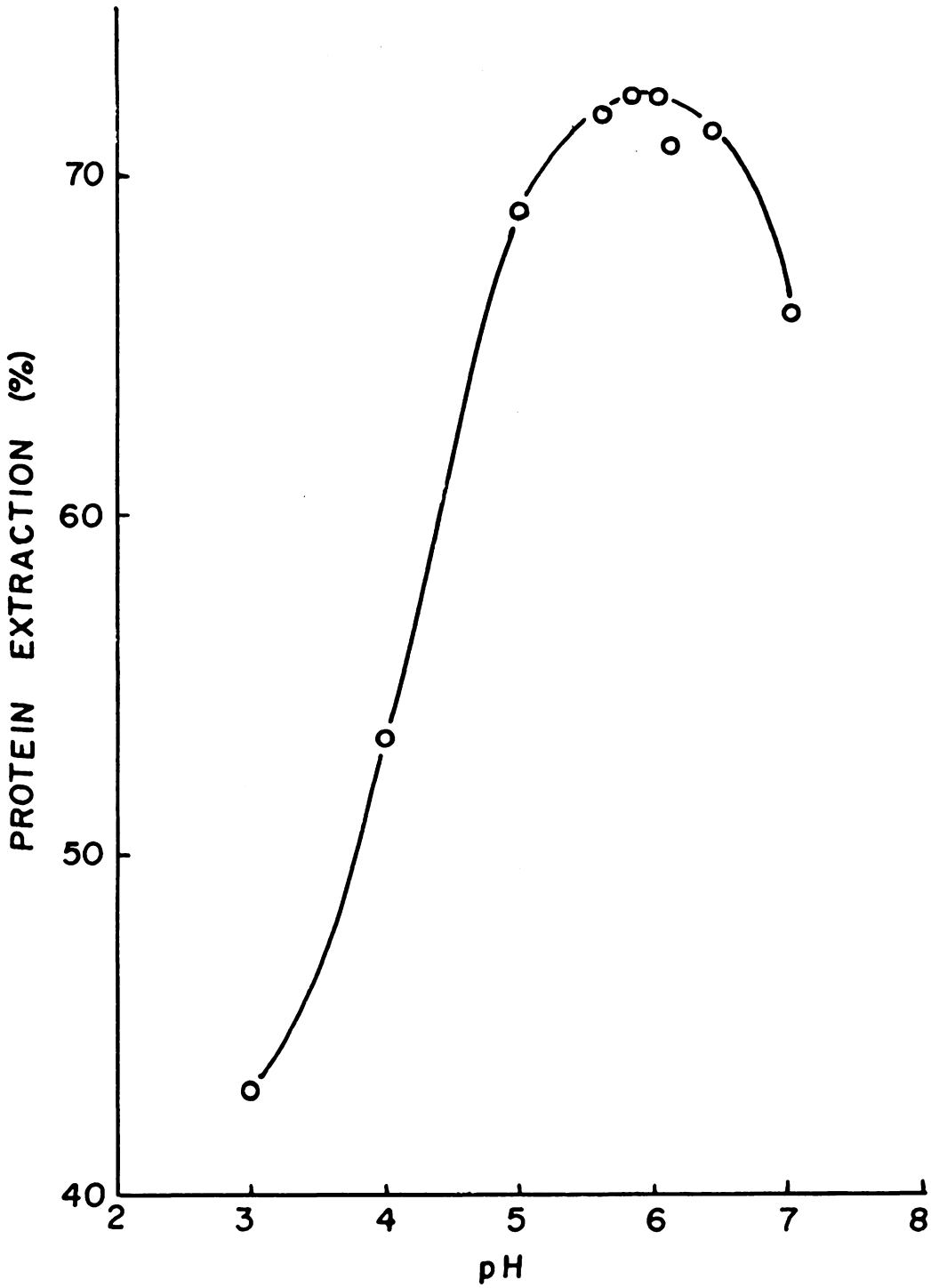

PROTEIN EXTRACTION (%)
70
60
50
40
2
3
4
5
6
7
8
pH

Table 11

Determination of the Effect of Concentration of P. westerdijkii Enzyme on Protein Extraction *

Volume of Enzyme Used (ml)	Protein Extraction (%)
0.0	71.6
10.0	77.6
20.0	82.0
30.0	83.6
40.0	85.0

*Different volumes of enzyme were added to 2 gm of coconut flour, the final volume was made up to 40 ml in a 100 ml beaker, and they were incubated for six hours at 40°C. The milk was extracted and the crude protein content was determined as mentioned in Table 3.

Table 12

Determination of the Effect of Incubation Time on Protein and Dry Matter Extraction by *P. westerdijkii* Enzyme*

Volume of Enzyme (ml)	Incubation Time (hrs.)							
	3		24		48		72	
	P.E.(%)	D.E.(%)	P.E.(%)	D.E.(%)	P.E.(%)	D.E.(%)	P.E.(%)	D.E.(%)
0.0	7.7	15.5	13.0	16.1	---	14.3	---	14.4
5.0	26.0	19.5	41.3	25.9	49.6	29.2	54.6	32.9
10.0	32.3	21.6	57.6	30.8	63.3	36.7	67.6	43.1
15.0	36.0	23.0	62.0	34.5	68.0	41.5	68.0	47.7

P.E.: Protein Extraction D.E.: Dry Matter Extraction

*One gm of coconut residue plus 5, 10, or 15 ml of partially purified enzyme adjusted to pH 6.0 were placed in 100 ml beakers and the volume was made up to 50 ml and incubated at 40°C for 3, 24, 48 and 72 hours. A few drops of toluene were added to prevent bacterial growth. After the specified period of incubation the pH of the contents of the beaker was raised to 7.0 with dilute NaOH and further incubated for 30 minutes at room temperature. Then the contents of the beaker were filtered through Whatman No. 1 filter paper and the nitrogen content of the filtrate was determined by the microkjeldahl procedure and converted into crude protein, using a conversion factor of 6.25. The residue was dried in a hot-air oven at 100°C for 24 hours and weighed.

Fig. 5. Determination of the Effect of Incubation Time on Protein Extraction by P. westerdijkii Enzyme

0,5,10 and 15 ml refer to volume of enzymes per gram of coconut residue.

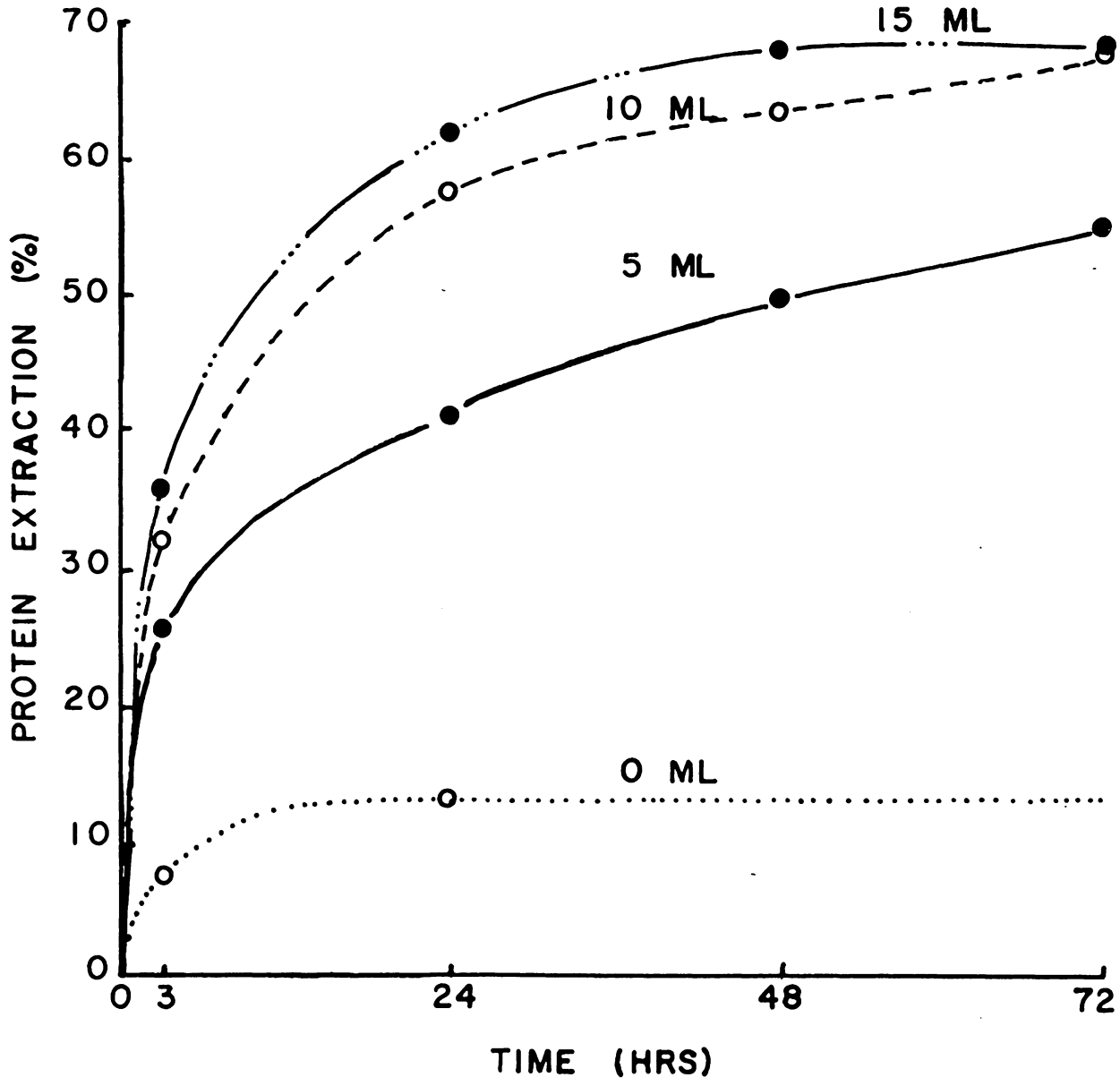
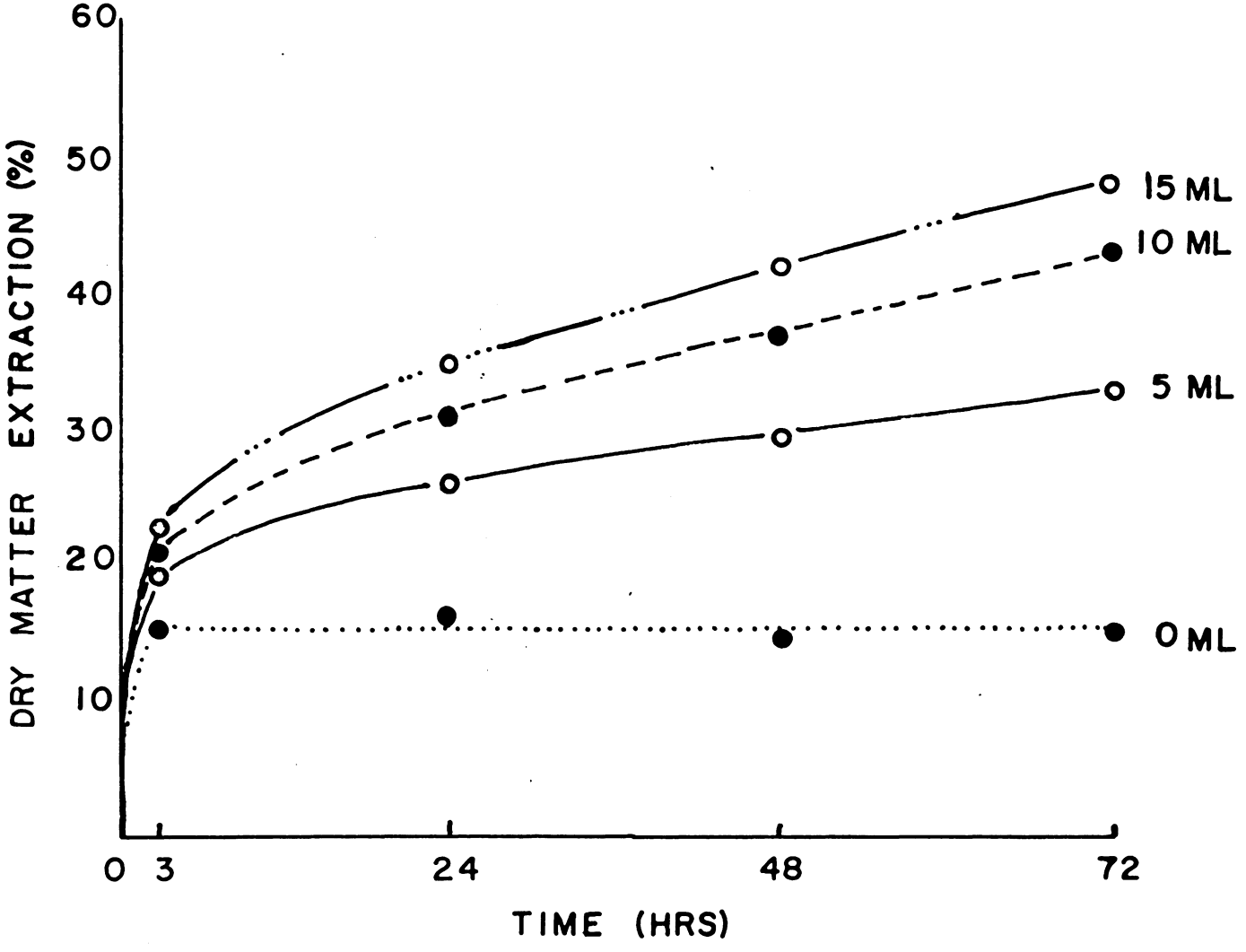

70
60
50
40
30
20
10
0
PROTEIN EXTRACTION (%)
15 ML
10 ML
5 ML
0 ML
0
3
24
48
72
TIME (HRS)

Fig. 6. Determination of the Effect of Incubation Time on Dry Matter Extraction by P. westerdijkii Enzyme

0,5,10 and 15 ml refer to volume of enzymes per gram of coconut residue.


60
50
40
30
20
10
DRY MATTER EXTRACTION (%)
15 ML
10 ML
5 ML
0 ML
0
3
24
48
72
TIME (HRS)

24 hours. Only slight improvement was noticed over 72 hours incubation.

8. Processing of Coconut Flour for Rat Feeding Experiments.

In large scale preparations 84.5 and 79.2 percent extractions of protein and dry matter were observed by enzymatic-treatment. Table 13 shows the achievement of 70 percent protein extraction over the control in the above case. The percentage of protein extraction from coconut flour varied even among the controls in bench and large scale preparations.

Table 14 shows the material balance on processing 1200 gm of coconut flour. The total crude protein and crude fiber values for control, boiled-enzyme-treated, and enzyme-treated preparations are in excellent agreement. It is evident from the table that more than half the amount of residue (non-extractable dry matter) in the control was solubilized by enzyme treatment. The material balance for ash, and ether extract was also very close in all the above preparations.

9. Amino acid Analysis.

The amino acid analyses are given in Table 15. The total amino acid content did not add up to 100 percent of the crude protein in any case. However, the total amino acid contents are almost equal for all the coconut milk preparations. On one hand the total amino acid figures for control and boiled enzyme-treated residues are 72.07 and 72.12 respectively; on the other hand, the enzyme-treated residue amino acids summed up to 90.42, indicating the presence of very little other nitrogenous material.

Table 13

Processing of Coconut Flour for Rat Feeding Experiments
(i) Comparison of Protein and Dry Matter Extraction with and without P. westerdijkii Enzyme Treatment*

Description	Protein Extraction (%)	Dry Matter Extraction (%)
Control (no enzyme)	49.4	59.5
Control (boiled enzyme)	54.6	59.8
Enzyme-treated	84.5	79.2

*Each 100 gm of coconut flour was suspended in 800 ml of distilled water and incubated at 40°C. After three hours the contents were transferred from the beaker to a large stainless-steel Waring blendor and blended for 4 minutes. The milk was expressed through four layers of cheese cloth. The residue was again suspended in 500 ml of distilled water or 400 ml of enzyme adjusted to pH 6.0 (8 ml enzyme/gm of dry residue) plus 100 ml of distilled water and again incubated at 40°C for three hours; at that time the contents were transferred to the Waring blendor, blended for two minutes, and expressed through four layers of cheese cloth to collect the milk. The residue was re-suspended in 200 ml of distilled water, allowed to stand at room temperature for 30 minutes and the milk was collected as before. The milk preparations were then pooled together, and filtered through four layers of cheese cloth once again. The milk preparation and residue were lyophilized in trays.

Table 14

(ii) Material Balance on Processing 1200 gm of Coconut Flour

Preparation	Dry Matter (gm)		Protein (gm)		Crude Fiber (gm)	
	E.	N.E.	E.	N.E.	E.	N.E.
Control (no enzyme)	486	596	139	118	9	169
Control (boiled enzyme)	482	598	153	115	13	187
Enzyme-treated	835	250	237	26	19	100

E.: Extractable N.E.: Not-extractable

The weight percent of total amino acids are shown in Table 16, and reveal either significant or slight increases in the extraction of the following essential amino acids in enzyme-treated compared to conventional milk: threonine, valine, methionine, isoluecine, leucine and phenylalanine; the enzyme-treated residue shows corresponding decreases in the above amino acids, thus confirming the ability of the enzyme to strip out the protein from the coconut flour.

Tables 17 and 18 show the comparison of the amino acid requirements of the rat (33) and the amino acid composition of various coconut preparations.

It is quite obvious from Table 18 that the most limiting amino acid in the coconut milk preparation is lysine in confirmation of Butterworth and Fox (4), and Thomas and Scott (41), followed by threonine, the sulfur amino acids and isoleucine. Enzyme-treatment caused significant improvement in threonine and isoleucine and the order of limitation became lysine, the sulfur amino acids, isoleucine, and lastly, threonine.

Due to the fact that lysine and sulfur amino acids are relatively inexpensive, they could be used to supplement diets prepared from the above preparations. It is worth mentioning that the enzyme could be activated by using cysteine hydrochloride which not only improved extractability of protein from coconut flour but also would tend to balance the sulfur amino acid deficit.

10. Evaluation of Protein Quality.

(i) Determination of PER: The PER obtained for control

Table 15

Amino Acid Composition of Coconut Milk and Residue Preparations (gm/100 gm crude protein)*

Amino acid	C.M.	C.R.	B.M.	B.R.	E.M.	E.R.
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

*Analysis was done according to Spackman et al.
Internal standards: Nor-leucine and β-guanidino-alanine with long and short columns respectively.

Symbols: C.M.: Control Milk; C.R.: Control Residue; B.M.: Boiled Enzyme treated Milk; B.R.: Boiled Enzyme treated Residue; E.M.: Enzyme treated milk; E.R.: Enzyme treated Residue

Table 16

Amino Acid Composition of Coconut Milk and Residue Preparations (% of total amino acids)*

Amino acid	C.M.	C.R.	B.M.	B.R.	E.M.	E.R.
Lysine	4.5	4.7	5.1	5.6	4.5	4.7
Histidine	2.1	2.0	1.9	2.5	2.0	2.2
Ammonia	1.8	2.2	2.0	2.3	2.2	2.2
Arginine	15.9	13.4	15.1	16.9	14.2	12.3
Aspartic acid	8.6	11.7	9.0	11.8	8.8	10.4
Threonine	2.9	4.3	3.5	4.4	3.7	4.3
Serine	4.2	6.1	2.8	5.4	4.4	5.0
Glutamic acid	20.7	6.4	20.1	5.2	19.4	16.3
Proline	3.6	4.5	3.8	4.6	3.9	4.1
Glycine	4.7	5.8	5.0	6.0	5.0	5.3
Alanine	3.9	4.9	3.9	5.0	4.2	4.3
Half-cystine	1.9	1.1	2.2	1.1	1.6	1.3
Valine	5.4	7.4	5.6	5.9	5.7	6.2
Methionine	1.1	1.2	0.9	1.4	1.2	0.7
Isoleucine	3.7	5.2	4.1	5.3	4.0	4.4
Leucine	6.7	9.0	7.0	9.4	7.0	7.7
Tyrosine	2.8	3.0	2.7	3.0	2.8	2.8
Phenylalanine	4.4	6.0	4.4	6.1	4.6	4.8

*Note tryptophan is not included.

Symbols: C.M.: Control Milk; C.R.: Control Residue; B.M.: Boiled Enzyme treated Milk; B.R.: Boiled Enzyme treated Residue; E.M.: Enzyme treated milk; E.R.: Enzyme treated Residue

Table 17

Comparison of Amino Acid Composition of Various Coconut Preparations with the Requirements of the Rat

Amino acid	% of Diet with 10% True Protein									
	Requirement of rat		Coconut Milk			Coconut Residue				
			Control		Enzyme Treated		Control		Enzyme Treated	
Histidine	0.25		0.21		0.20		0.20		0.22	
Lysine	0.90		0.45		0.45		0.47		0.47	
Tryptophan	0.11		---		---		---		---	
Isoleucine	0.55		0.37		0.40		0.52		0.44	
Valine	0.55		0.54		0.57		0.74		0.62	
Leucine	0.70		0.67		0.70		0.90		0.77	
Threonine	0.50		0.29		0.37		0.43		0.43	
Methionine	0.16		0.11		0.12		0.12		0.07	
		0.50		0.30		0.28		0.23		0.20
Cystine	0.34		0.19		0.16		0.11		0.13	
Phenylalanine	0.42		0.44		0.46		0.60		0.48	
		0.72		0.72		0.74		0.90		0.76
Tyrosine	0.30		0.28		0.38		0.30		0.28	

Table 18

Amino acid Composition of Coconut Preparations Expressed as percent of the Requirement Estimated by Rama Rao et al. (33)

Amino acid	% of Requirement			
	Coconut milk		Coconut Residue	
	Control	Enzyme treated	Control	Enzyme treated
Histidine	84.0	80.0	80.0	88.0
Lysine	50.0	50.0	52.2	52.2
Tryptophan	--	--	--	--
Isoleucine	67.3	72.7	94.5	80.0
Valine	98.2	103.6	134.5	112.7
Leucine	95.7	100.0	128.6	110.0
Threonine	58.0	74.0	86.0	86.0
Methionine Cystine	60.0	56.0	46.0	40.0
Phenylalanine Tyrosine	100.0	102.8	125.0	105.6

flour was 0.48 as against the PER of 2.2 obtained with a previous batch of flour. This suggests that the quality of protein in this batch was inferior probably because of heating during its manufacture at CFTRI. The PER for control and boiled-enzyme-treated milks were almost the same, thus eliminating possible enzyme toxicity (0.80 and 0.71 respectively). The enzyme-treated-milk gave a low PER, almost equal to that of coconut flour (0.47). It is worth mentioning here that the rats in all coconut milk groups showed marked gain in weight during the last three days of the experimental period suggesting that the animals might have undergone the stress of adjusting themselves for the new food. So it would be of more value if the experimental period were prolonged.

Even though there is a large apparent difference between the PER's of control and enzyme-treated milk groups in the raw data, Chi-square and simple T-test analyses revealed no statistical significance between the PER's shown in Table 19.

The animals on the residue groups all lost weight but those which were on enzyme-treated residue did very poorly; the rats had a bloated appearance and lost balance during the end of the experimental period. The effect might be due to the sub-optimal level of essential amino acids present in the enzyme-treated residue compared to the other two residue preparations (Table 19).

(ii). Determination of Apparent Digestibility. The behavior of the animals in the above experiments suggested an experiment on the digestibility of the various proteins. Table 20 shows that the

protein digestibility is low for the diets composed of coconut residue compared to coconut milk. It is even lower for enzyme-treated residue. Hence, it is quite obvious that the poor digestibility of the diets restricted the protein intake, the effect of which was reflected by the weight loss of the rats.

11. Enzymological Observations

1. Product Analysis

Incubation of partially purified enzyme (7 units of C_1/ml)and hydrocellulose (5 mgs/ml) at pH 6.0 and 40°C resulted in about 77 percent hydrolysis of hydrocellulose. The results of the chromatographic analysis of the hydrolysate through the Miller column can be seen in Table 21. Low recovery, the clogged appearance of the column at the higher gradient of aqueous ethanol and the decrease in the volume of the fractions collected at this stage suggested the precipitation of higher oligosaccharides of D.P. 10 and above. The D.P. analysis of the hydrolysate revealed an average D.P. of 11 confirming the above suggestion. Table 21 also indicates that the enzyme is more capable of handling cellobiose through cellohexaose than the higher oligosaccharides to form glucose.

2. Identification of Water Soluble Reducing Sugars in the Coconut Milk Preparations

According to Caray (5) copra meal (coconut flour) contains sucrose, raffinose, galactose, glucose, fructose, pentoses, cellulose, pentosans, starch, dextrin and galactan. Paper chromatography of the various coconut milk preparations revealed the presence of arabinose, glucose, galactose and disaccharides. However, the

Table 19

Evaluation of Protein Quality
(i) Determination of PER*

Dietary Protein Source	No. of Rats	No. of Days	Wt. Change Average/Rat (gm)	PER
Control Milk (no enzyme)	9	10	+ 5.0	0.80
Control Milk (boiled enzyme)	10	10	+ 4.2	0.71
Enzyme Treated Milk	10	10	+ 2.6	0.47
Casein I[1]	10	10	+28.1	3.40
Casein II[2]	10	7	+14.1	2.11
Coconut flour	10	7	+ 3.0	0.48
Control Residue (no enzyme)	10	7	- 1.8	
Control Residue (boiled enzyme)	9	7	- 2.2	
Enzyme Treated Residue	4	7	- 5.2	

*The diets were formulated to contain 10 percent cottonseed oil, 5 percent salt, one percent vitamin mixture, 10 and 9 percent protein, and 3 percent or 30.6 percent crude fiber in the case of milk and residue preparations respectively. The crude fiber level was made up with solka-floc wherever it was necessary.

Salt mixture and vitamin mixture excluding vitamins A, D, and E, were prepared according to Campbell's Procedure (2). Vitamins A,D, and E mixture were prepared using Percomorph and α-Tocopherol. Each 100 gm of diet contained 208, 90, and 6.6 I.U. of vitamins A, D, and E respectively.

Twenty-one day old weanling male rats of the Sprague-Dawley strain weighing 55 ± 10 gm were used. The rats were kept in individual wire-screen cages. Food and water were given ad libitum. The experiments lasted 7 and 10 days for residue and milk preparations respectively.

Even though there is a large difference between the PER's of control and enzyme-treated milk groups in the raw data, Chi-square and simple T-test analyses revealed no statistical significance between the PER's shown in the table.

[1]Control for coconut milk groups. [2]Control for residue groups and coconut flour group.

Table 20

(ii). Determination of Apparent Digestibility*

Dietary Protein Source	Apparent Digestibility (%)
Control Milk (no enzyme)	82.5
Enzyme treated milk	76.1 (n.s.)
Control Residue	62.1 (**)
Enzyme treated Residue	43.6 (**)

*Sprague-Dawley strain male rats were individually kept in metabolism cages. Food and water were given ad libitum. The food intake was measured every day. The nitrogen content of urine, collected under toluene, and the feces was determined by the A.O.A.C. macrokjeldahl method (1).

(n.s.) not significantly different from control milk

(**) highly significantly different from control milk ($p \leq .01$).

Table 21

(i). Identification of Hydrolytic Products from Hydrocellulose by P. westerdijkii Enzyme*

Sugar	Glucose Equivalent (α)	% of Total Soluble Sugar	% of Recovered Sugar
Glucose	260	12.10	32.74
Cellobiose	26	1.21	3.27
Cellotriose	14	0.65	1.76
Cellotetraose	11	0.51	1.38
Cellopentaose	20	0.93	2.51
Cellohexaose	16	0.73	2.01

*Three ml of hydrocellulose (5 mg/ml) plus 20 ml of partially purified enzyme were adjusted to pH 6.0 and placed in a 100 ml Erlenmeyer flask with a few drops of toluene to prevent bacterial growth. The flask was incubated at 40°C for 120 hrs. The contents of the flasks were autoclaved at 121°C for 20 minutes and filtered through Millipore filters. The filtrate was boiled for 30 minutes and again filtered through Millipore filters. The filtrate (7.5 ml) was concentrated in a rotary evaporator to one ml of which 0.5 ml was put on Miller column and eluted with a gradient of aqueous ethanol to collect 2 ml fractions. Total sugar and average D.P. were determined according to Timell's procedure (40).

amounts of these sugars varied a great deal in different preparations as can be seen in Table 22. The marked increase in arabinose, glucose and galactose in the enzyme treated milk indicates primary degradation of the pentosans, glucans such as starch, dextrin and/or cellulose and galactan by the enzyme. These results are more meaningful if compared with the reduction of non-extractable crude fiber shown in Table 18, for they establish that the enzyme accomplishes a net reduction in structural polysaccharides.

3. Degradation of Filter Paper and Other Carbohydrates.

The ability of the enzyme to degrade pieces of Whatman No. 1 filter paper at various concentrations can be seen in Plate 1. Table 23 shows a weight loss of 43 percent at the highest concentration of the enzyme. The gradual decrease in weight loss corresponding to the decrease in the units of C_1 activity is noteworthy. Toyama (42) developed a method for measuring cellulose activity by observing the time in seconds required for breakdown of filter paper.

Table 24 shows the capacity of the enzyme to hydrolyse various carbohydrates, and establishes quite a variety of substrate specificities. The loss of enzyme activity observed during gel filtration (Fig. 1) might be due to the adherence of the enzyme to the gel.

4. Vegetable Processing

The results of the enzymic action on various vegetable food stuffs can be seen in Plates 2,3,4,5,6,7,8,9,10 and 11. Cucumber, radish, carrot, apple, onion, potato and shredded coconut were completely

Plate 1. Degradation of Filter Paper by P. westerdijkii Enzyme

Pieces of Whatman No. 1 filter paper weighing 27 mgm were incubated at 40°C for 48 hours with varying concentrations of enzyme except for the tube at right which contained no enzyme. The tube at left labeled 1, contained 28 units of C_1 in 4 ml of distilled water. Dilutions of enzyme in the other tubes were as indicated.


1
1/2
1/4
1/8
1/16
1/32
C

Table 22

(ii). Determination of Water Soluble Reducing Sugars in Various Coconut Milk Preparations*

Sugar	Control Milk (no enzyme)	Boiled Enzyme-treated Milk		Enzyme-treated Milk	
		mg/gm	Increase over Control	mg/gm	Increase over Control
Arabinose	3.7	3.3	None	30.0	26.3
Glucose	13.9	13.8	None	36.6	22.7
Galactose	11.3	12.2	0.9	17.2	5.9
Disaccharides	1.3	1.3	None	1.3	None

*One hundred mg of various milk preparations (dry matter) were dissolved in one ml distilled water. Two Whatman No. 1 filter papers were spotted with eight, eight, and five .01 ml spots of control, boiled enzyme-treated, and enzyme-treated milks. The papers were developed for 20 hrs. in pyridine-n-butanol-water (6:4:1), dried and one paper was sprayed with the periodate-benzidine spray (6). Various spots were located and the corresponding areas were cut from unsprayed paper and each strip was eluted with 5 ml of distilled water for 2 hrs. and one ml. portions were used to analyze the reducing sugar content following the procedure of Nelson and Somogyi (26, 38).

Table 23

Enzymatic Degradation of Filter Paper-Estimation of Hydrolytic Activity at Different Concentrations of Partially Purified *P. westerdijkii* Enzyme*

Units of C_1 Activity/tube	Weight loss (%)
28.00	43.0
14.00	18.9
7.00	6.6
3.50	4.0
1.75	4.7
0.88	2.9

*Pieces of Whatman No. 1 filter paper, 8 x 40 m.m., were incubated with 4 ml of enzyme (adjusted to pH 6.0) at a series of dilutions in 18 x 150 m.m. test-tubes and incubated at 40°C for 48 hrs. At the end of incubation period, the tubes were gently shaken. Toluene was used to prevent bacterial growth. Then the contents of the tubes were dried in a hot air oven and weighed.

Table 24

Testing the Hydrolytic Activity of P. westerdijkii Enzyme on Various Carbohydrates*

Substrate	Susceptibility to Hydrolysis
β-1-4-glucans	
Hydrocellulose	+
Cellobiose	+
Cellotriose	+
Cellotetraose	+
Cellopentaose	+
Cellohexaose	+
Substituted β-1-4-glucans	
CMC-70-L=	+
CMC-70-M=	+
Cellobiose octaacetate	-
Other β-glucosides	
Esculin	+
p-Nitrophenyl- β-D-Glucopyranoside	+
Salicin	+
Amygdalin	+
Gentiobiose	+
Other Carbohydrates	
β-D-Glucose pentaacetate	±
Gum arabic	+
Inulin	+
Maltose	+
Melebiose	+
Melezitose	-
Trehalose	±
Turanose	+
Gum ghatti	+
Lactose	+
Raffinose	+

Table 24 Continued

Substrate	Susceptibility to Hydrolysis
Sucrose	+
Sephadex G-25	-
Sephadex G-75	-
Sephadex G-100	-
Biogel P-10 (Polyacrylamide)	-
Amylopectin	+
Amylose	+
Polygalacturonic acid	+
Detran	-
Xylan (P)	+
Pectin	-
Pectic acid	±

Symbols: +: Clearcut evidence of Hydrolysis
-: No detectable Hydrolysis
±: Hydrolytic products, if produced, were present in amounts making detection questionable.

*One mg of the substrate was dissolved in 0.05 ml distilled water in a 1 3/4 x 3/8" test-tube to which 0.05 ml of partially purified enzyme adjusted to pH 6.0 was added. After mixing well 0.005 ml portions of the reaction mixture were spotted on a Whatman No. 1 filter paper. The tube was incubated at 40°C for one hour and 0.005 ml portions of the reaction mixture were again withdrawn and spotted on the paper (20). Chromatograms were developed in the ethylacetate-pyridine-water solvent of Jermyn and Isherwood (15) for 20 hrs. and the products were identified by periodate-benzidine spray (6).

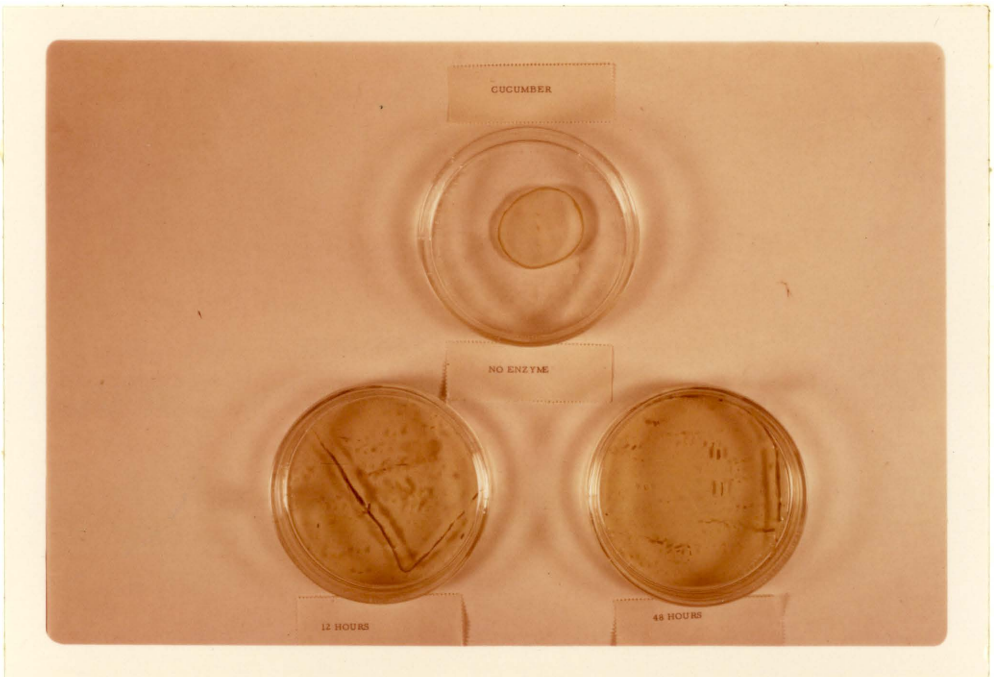
solubilized except for the outer peels in some instances within 48 hrs. when incubated with 4 to 6 ml of enzyme (7 units of C_1 activity/ml) at pH 6.0 and 40°C. The section of orange fell into pieces consisting of individual cells during that incubation time. The slight degradation that resulted with the whole green bean and the piece of cabbage can be attributed to the presence of the outer, waxy cuticle layer which acts as a barrier to the enzyme's reaching the internal cell parts. Noticeable degradation occurred within 12 hrs. of incubation. Similar results were reported by Toyama (43, 45) with the enzyme preparations obtained from Trichoderma spp., Aspergillus niger, and A. oryzae.

Plate 2. Complete solubilization of Cucumber by P. westerdijkii Enzyme

Sections of Cucumber approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.

Plate 3. Complete solubilization of Radish by P. westerdijkii Enzyme

Sections of Radish approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.


CUCUMBER
NO ENZYME
12 HOURS
48 HOURS

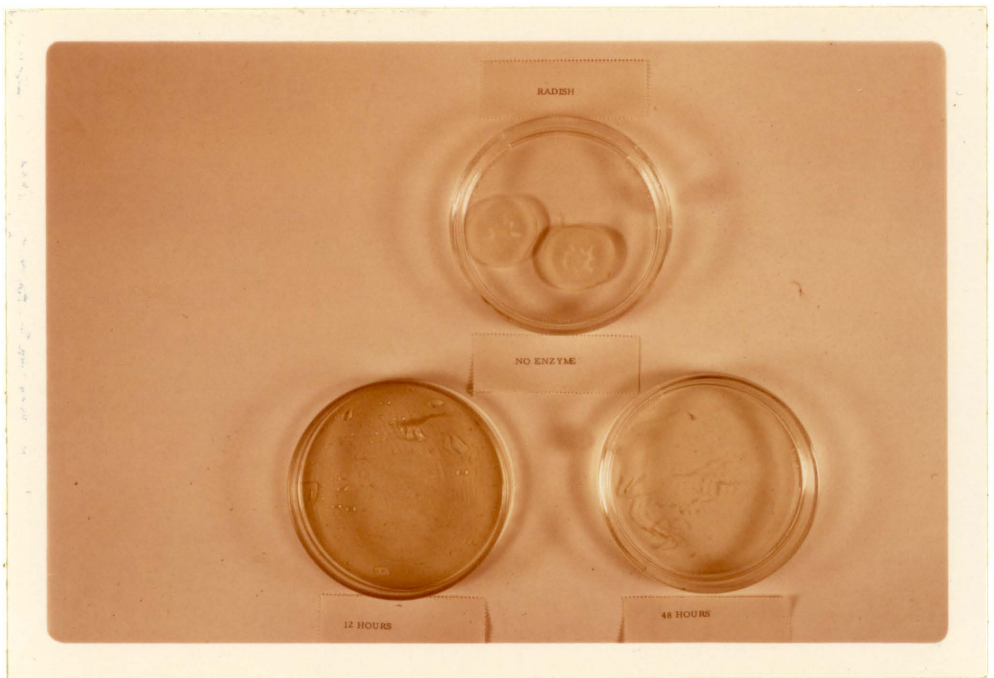

RADISH
NO ENZYME
12 HOURS
48 HOURS

Plate 4. Complete solubilization of carrot by P. westerdijkii Enzyme

Sections of Carrot approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.

Plate 5. Complete solubilization of Apple by P. westerdijkii Enzyme

Sections of Apple approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.


CARROT
NO ENZYME
12 HOURS
48 HOURS

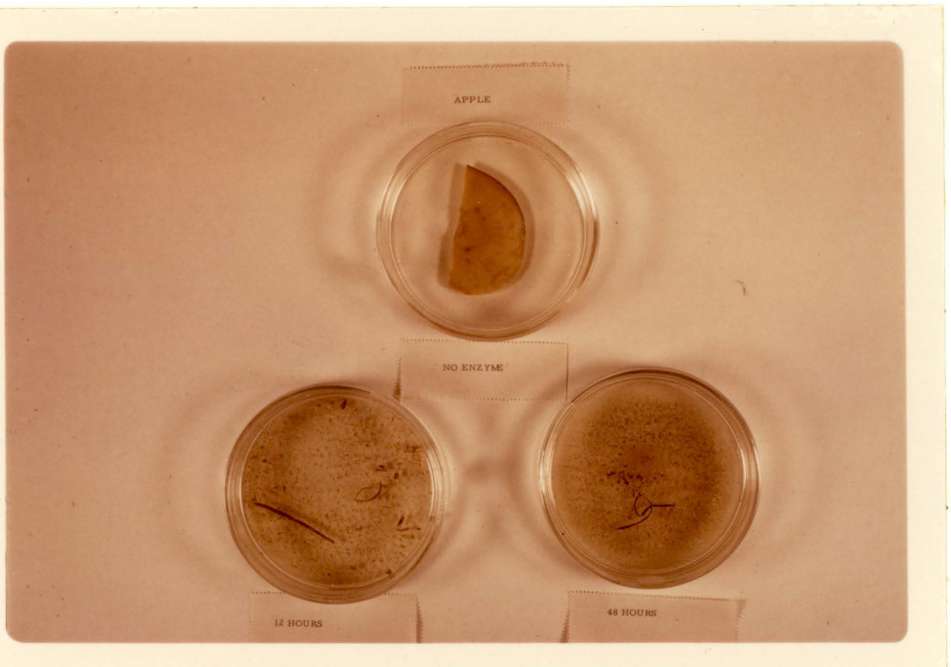

APPLE
NO ENZYME
12 HOURS
48 HOURS

Plate 6. Complete solubilization of Onion by P. westerdijkii Enzyme

Sections of Onion approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.

Plate 7. Complete solubilization of Potato by P. westerdijikii Enzyme

Sections of Potato approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.


ONION
NO ENZYME
12 HOURS
48 HOURS

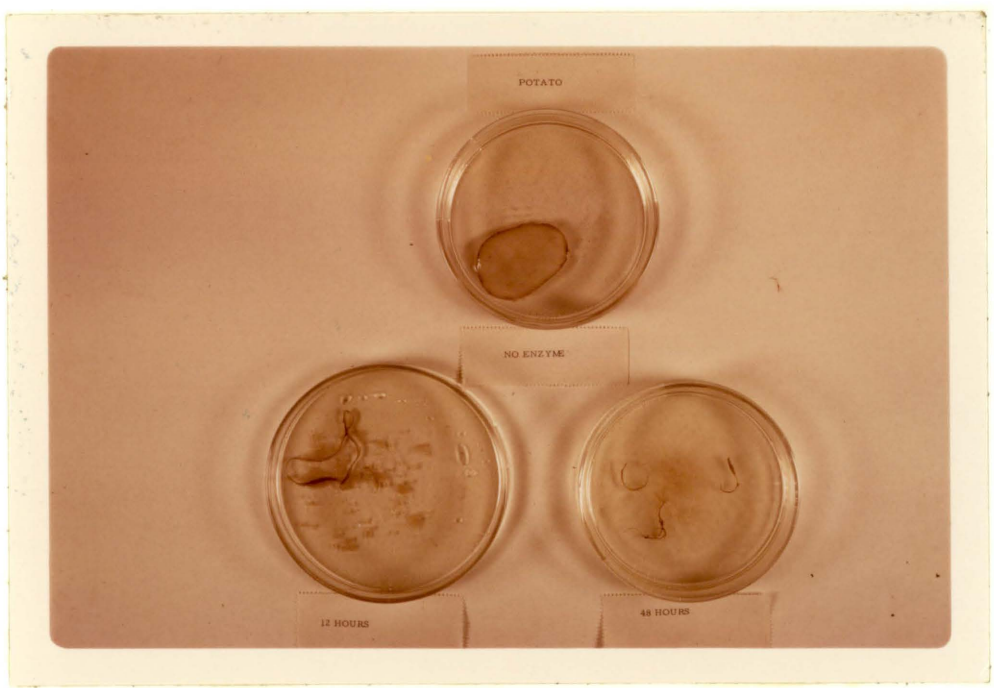
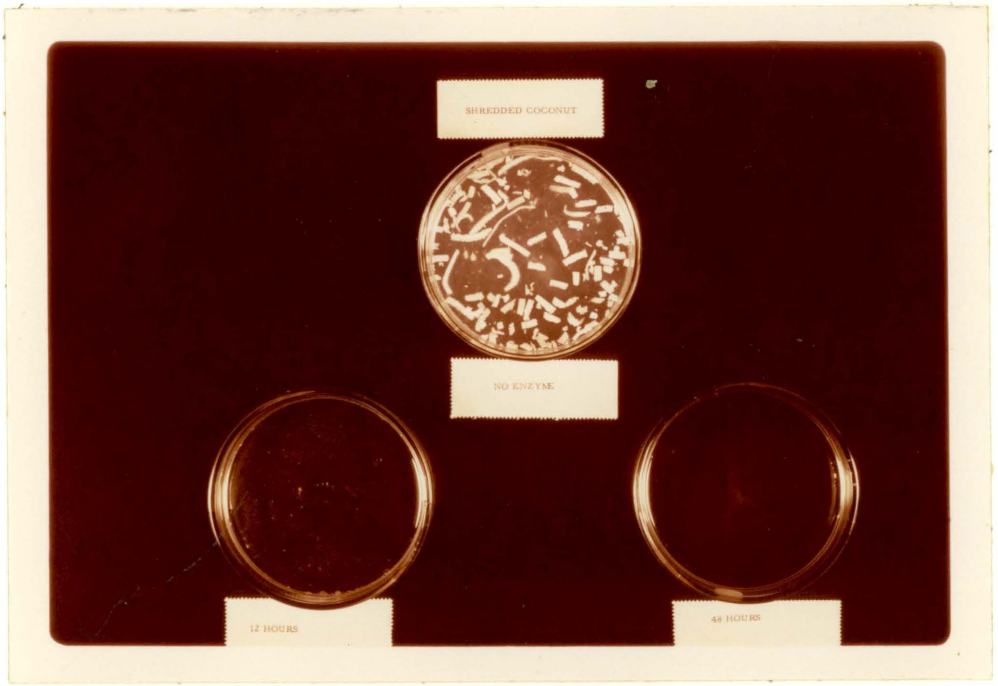

POTATO
NO ENZYME
12 HOURS
48 HOURS

Plate 8. Complete solubilization of Shredded Coconut by *P. westerdijkii* Enzyme

Plate 9. Effect of *P. westerdijkii* Enzyme on the section of Orange


SHREDDED COCONUT
NO ENZYME
12 HOURS
48 HOURS

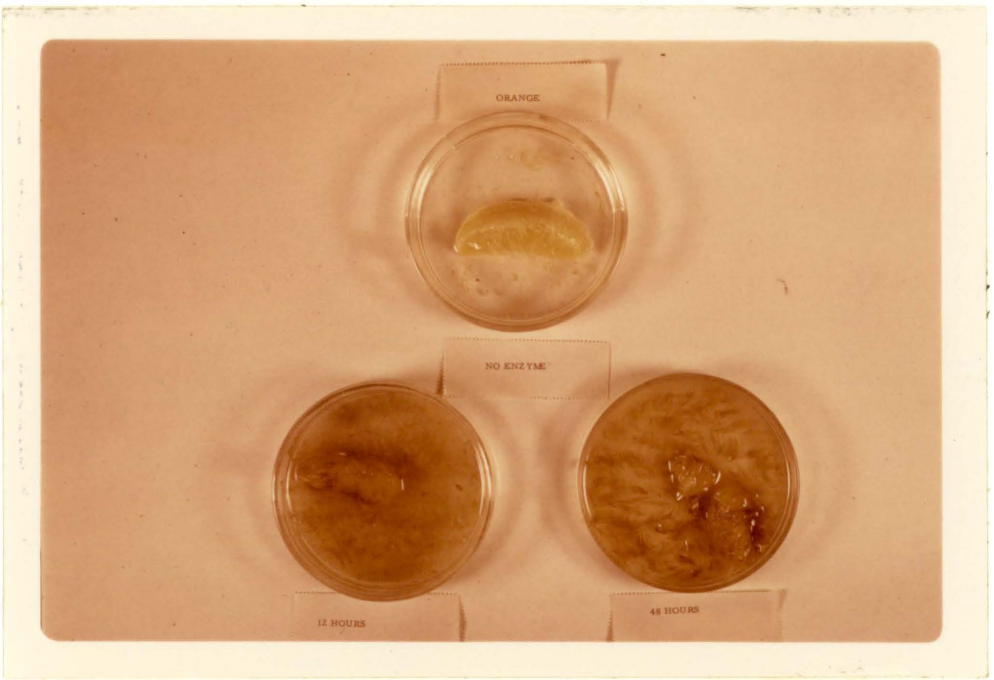
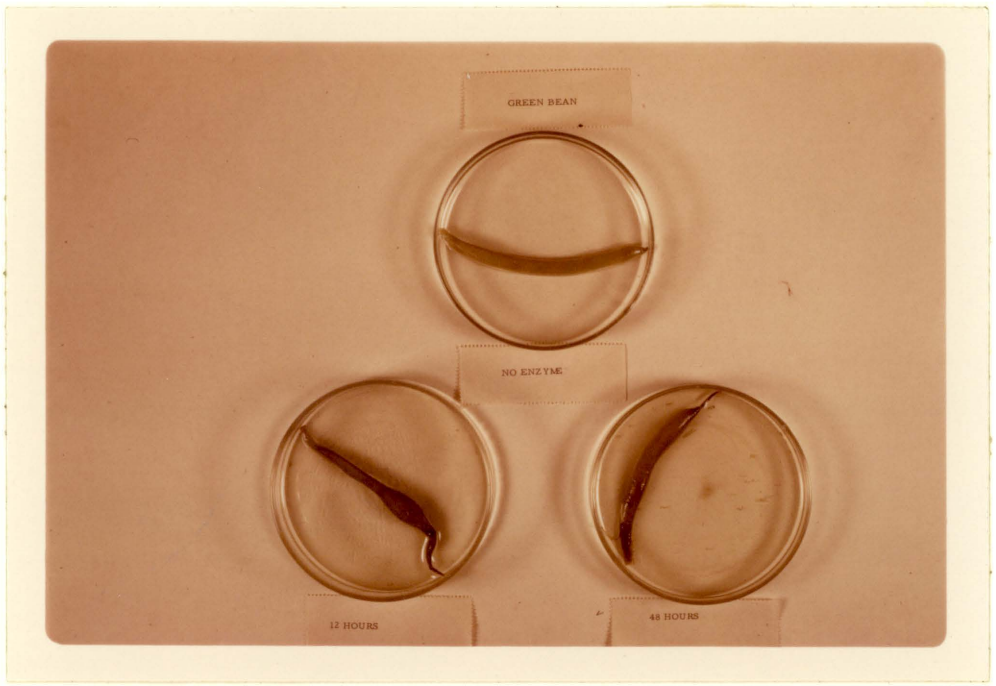
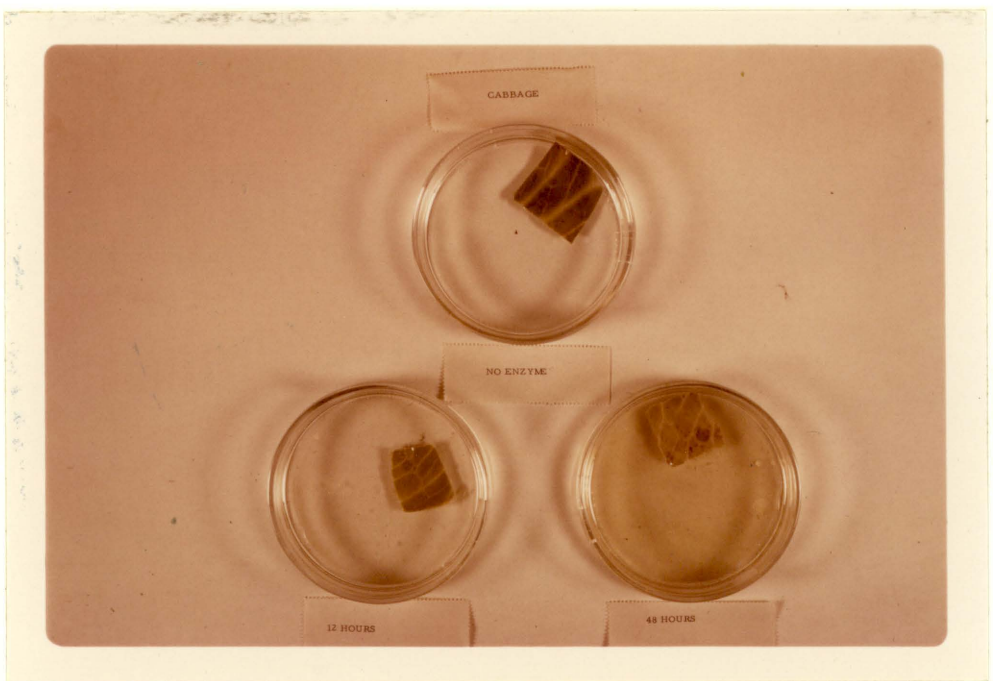

ORANGE
NO ENZYME
12 HOURS
48 HOURS

Plate 10. Effect of P. westerdijkii Enzyme on the whole Green Bean

Plate 11. Effect of P. westerdijkii Enzyme on the piece of Cabbage

Sections of Cabbage approximately two mm thick were incubated for 12 or 48 hours with 6 ml of enzyme (42 units of C_1) at 40°C. Control sections were incubated in 6 ml of water for 48 hours.


GREEN BEAN
NO ENZYME
12 HOURS
48 HOURS


CABBAGE
NO ENZYME
12 HOURS
48 HOURS

V. SUMMARY AND CONCLUSION

1. Based on C_1 and CMC-ase activities, *A. terreus*, *Basidiomycetes* sp., *C. globosum*, *M. verrucaria*, *P. westerdijkii*, and *T. viride* were screened for enzyme production on coconut flour plus the trace mineral solution medium.

2. *P. westerdijkii* yielded highly active enzyme when grown on coconut flour-solka-floc (2:1) plus the trace mineral solution or tap water medium, at 31°C for 11 days.

3. The optimal conditions for the enzyme to cause maximum extraction of protein from coconut flour were pH 6.0 and 40°C in three hour test periods. Cysteine hydrochloride showed an activation effect on the enzyme under the above conditions.

4. A noticeable increase in protein and dry matter extraction occurred when coconut flour was treated with partially purified *P. westerdijkii* enzyme. The material balances on processing 1200 gm coconut flour for animal feeding experiments are in excellent agreement.

5. Partially purified *P. westerdijkii* enzyme showed a wide substrate specificity including the complete degradation of most of the vegetable foodstuffs tested.

The results discussed in this dissertation prove that it is technically feasible to reduce the crude fiber content of the coconut flour with a corresponding increase in protein extraction by treatment of the flour with *P. westerdijkii* enzyme. The enzyme appears to be non-toxic.

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*Original not seen.

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ABSTRACT

Postalotiopsis westerdijkii, a cellulolytic mold, exhibited the production of a freely diffusing, extra-cellular enzyme capable of solubilizing hydrocellulose and amorphous cellulose when grown on coconut flour-solka-floc (2:1) medium at 31°C for 11 days. For the first time, the pH and temperature optimum for the maximum activity of the enzyme was established. The potency of the enzyme increased by serial culturing of the mold on the same medium. Activation of enzyme by cysteine hydrochloride was observed. Five-fold increases in specific activity with the elimination of most of the non-enzymic nitrogen was achieved by $(NH_4)_2SO_4$ precipitation and gel filtration of the crude enzyme.

The enzyme treatment of coconut flour brought marked improvement in protein extractability with a corresponding decrease in crude fiber. This action of the enzyme was further established by the increase of water-soluble reducing sugars in the enzyme-treated milk due to the degradation of structural polysaccharides.

The amino acid composition of the control residue predicted ample room for possible improvement of the amino acid pattern in the enzyme-treated milk. The most limiting amino acids in the coconut milk preparation is lysine in confirmation of Butterworth and Fox (4), and Thomas and Scott (41), followed by threonine, the sulfur amino acids and isoleucine. Significant amounts of threonine and isoleucine were stripped out of the control residue

by the enzyme treatment, and the order of limitation became lysine, the sulfur amino acids, isoleucine and lastly, threonine.

The animal feeding experiments did not show any significant difference between the protein qualities of the control and enzyme-treated milk preparations; however, the animals on the residue diets all lost weight. Those which were on enzyme-treated residue did very poorly. Low digestibility of the enzyme-treated residue reflected by the restricted protein intake due to high fiber content, and the presence of the sub-optimal levels of essential amino acids compared to the control residue resulted in great loss in weight, bloated appearance and loss of balance at the end of the experimental period. The enzyme appears to be non-toxic.

The enzyme at its optimal pH and temperature solubilized hydrocellulose yielding oligosaccharides with an average degree of polymerization of eleven. Also, the enzyme was more capable of handling cellobiose through cellohexaose than the higher oligosaccharides to form glucose. The wide substrate specificity exhibited by the enzyme resulted in extensive degradation of pieces of Whatman No. 1 filter paper and certain vegetable foodstuffs.