Enzymatic Modification of the Extractability of Protein from Soybeans, *Glycine max*

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Enzymes capable of improving the extractability of protein in soybeans from a control value of approximately 74 to 95% have been derived from *Pestalotiopsis westerdijkii.* The resulting soybean milk product appeared to be free of toxic components, and the protein was of a quality equal or superior to that of soybean milk prepared without enzymatic treatment. The enzyme was produced in tray cultures on a wheat bran–Solka floc support moistened with a mineral salt mixture. After incubation for 10 days at 31° C. the enzyme was extracted with water, precipitated with ammonium sulfate, and dialyzed by gel filtration. For improving the extractability of soybean protein nearly optimum conditions of treatment were established at 32 mg. of enzyme protein per gram of soybean residue incubated at pH 4.6 for 6 hours at 37° C. Approximately one half of the crude fiber was solubilized during the enzymatic treatment.

The nutritional quality of soybean protein, the current high levels of world soybean production, and the high economic value of soybean oil place the crop in a particularly conspicuous position as a means of alleviating part of the world's continuing protein deficit. In recent years, soybean protein extracts, generally referred to as soy milks, have found a valuable place in infant feeding here and abroad for patients unable to tolerate human or bovine milk as a result of genetic failures in the metabolism of galactose or of allergic reactions to milk proteins.

In most instances, existing processes for extracting soybean protein recover from 70 to 80% of the total protein (*6*, *12*, *15*, *16*, *22*), and the quality of the protein in the unextracted residue is superior to that of the soy milk protein (*6*). Any improvement in the efficiency of protein extraction would therefore be expected to offer the double benefits of improved yield of a product of superior quality.

Following the leads of Toyama (*20*) and Nisizawa and Ozaki (*13*), the present report concerns a demonstration that enzymes capable of facilitating the extraction of soybean protein can be produced from cultures of *Pestalotiopsis westerdijkii.*

Experimental Procedures

Production of Enzyme. The cultures evaluated for their capacity to produce useful enzymes were *Chaetomium globosum*, A.M. :459, *Aspergillus terreus*, Q.M. :72f, *Myrothecium verrucaria*, Q.M. :460, *Pestalotiopsis westerdijkii*, Q.M. :381, *Trichoderma viride*, Q.M. :6a, and *Basidiomycetes* sp., Q.M. :806.

Spore inoculations were made onto trays containing a 2-cm. layer of wheat bran–Solka floc (Brown and Co.) at a weight ratio of 2 to 1 moistened with a mixture of inorganic salts (*14*). The trays were covered with aluminum foil to prevent contamination and incubated at the temperatures indicated for 4 to 10 days.

Enzyme was then extracted from the cultures by using 500 ml. of distilled water per 100 grams of culture medium with periodic stirring over a period of 3 hours at room temperature. After filtration, the filtrates were brought to 30% saturation with $(NH_4)_2SO_4$ and centrifuged. The supernatant was then brought to 70% saturation with $(NH_4)_2SO_4$, and the resulting precipitate was collected by centrifugation. The precipitate was redissolved in one tenth of the original volume using distilled water and desalted on a gel-filtration column packed with Sephadex G-25. The fast-moving brown band emerging first from the gel-filtration column contained all of the aryl-β-glucosidase (*8*) and was used without further treatment.

Analytical Procedures. The turbidimetric cellulose assay of Li, Flora, and King (*10*) was modified by replacing the hydrocellulose with a finely dispersed suspension of the crude fiber fraction of soybeans. For this purpose, 1 gram of soybean meal was boiled for 1 hour in 100 ml. of a digestion mixture containing 80 ml. of glacial acetic acid, 80 ml. of concentrated nitric acid, 32 grams of trichloroacetic acid, and 720 ml. of distilled water. After exhaustive extraction with water the air-dried residue was ground to a fine powder in an agate mill. Assays for cellulase components were also conducted using carboxymethylcellulose (*7*) and *p*-nitrophenyl-β-glucoside (*7*) as substrates.

Micro-Kjeldahl analyses were carried out according to Johnson (*9*) and proximate analyses as described by the A.O.A.C. (*1*).

Sugar chromatography was carried out as described by Hash and King (*7*), and the method of Timell (*19*) was used to determine the degree of polymerization (number of monosaccharide units per molecule of oligosaccharide) of the products of enzymatic hydrolysis of soybean preparations.

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Amino acid analyses (*18*) were carried out using internal standards of norleucine on the long columns and β-guanidinoalanine on the short column. Hydrolyses of 100-mg. samples were carried out at 121° C. in 10 ml. of 6*N* HCl in sealed tubes with an atmosphere of N_2. Duplicate analyses were made of samples hydrolyzed for 24, 48, and 72 hours to allow correction for losses during hydrolysis. Tryptophan analyses (*5*) were run independently.

Soybean Preparations. Whole soybeans were soaked for 12 hours in 3.5 volumes of water, dehulled, and resuspended in 8.3 liters of distilled water per kilogram of dry beans. After blending for 10 minutes in a Waring Blendor, the homogenate was filtered through four layers of cheesecloth, yielding a filtrate (soy milk) and a filter cake (soybean residue). All drying was done by lyophilization.

Results

Enzyme Production. Although all of the cultures evaluated are actively cellulolytic under appropriate conditions, they differ markedly in their capacity to solubilize the crude fiber preparation derived from soybeans as seen in Table I. On the basis of these data, *Pestalotiopsis westerdijkii* was selected for further study because of its consistent activity on both soybean fiber in the turbidimetric assay (*10*) and carboxymethylcellulose in the reducing sugar assay (*7*).

Comparing the activity of enzyme produced by cultures grown on the wheat bran–Solka floc medium with that from cultures grown on a soybean residue medium resulted in 17 and 7% extraction of the soybean residue nitrogen, respectively. In this experiment, 1 gram of residue (42 mg. of N) was incubated with 10 ml. of enzyme at pH 4.0 and 37° C. for 6 hours in the presence of toluene. A nonenzymatic control was run in parallel and the nitrogen extracted here (2%) was subtracted before the values were calculated. Micro-Kjeldahl analyses on filtrates passing Whatman No. 1 filter paper were then carried out to establish the amount of protein extracted. On the basis of these results the wheat bran–Solka floc medium was used in all subsequent studies.

Table I. Enzyme Production by Several Cellulolytic Fungi[a]

Culture	Units of Enzyme per Milliliter: Soybean fiber substrate (*10*)	Units of Enzyme per Milliliter: Carboxymethylcellulose substrate (*7*)
Pestalotiopsis westerdijkii	1.0	16
Chaetomium globosum	0.8	9
Trichoderma viride	0.4	1
Basidiomycetes sp.	0	6
Myrothecium verrucaria	0	13
Aspergillus terreus	0	12

[a] Aqueous extracts from 10-day cultures grown at 28° C. were prepared using 500 ml. of water per 100 grams of culture. The precipitate obtained between 30 and 70% saturation with $(NH_4)_2SO_4$ was then redissolved in one tenth of the original volume of distilled water and assayed directly.

In experiments designed to estimate the effect of temperature on enzyme production, cultures were inoculated and incubated for 10 days at 27°, 31.5°, and 36° C. Growth occurred only at the two lower temperatures. One-gram portions of soybean residue were then incubated for 6 hours at pH 4.8 with 10 ml. of the enzyme processed as described earlier along with controls containing no enzyme. The enzyme preparations were adjusted to 1 mg. of protein per ml. Nitrogen extractions of 7.8 and 9.5% and weight losses of 8.7 and 19.3% were observed with enzymes from cultures grown at 27° and 31.5° C., respectively. In subsequent work incubation temperatures of 31° to 32° C. were used routinely.

In assessing the effect of culture age on enzyme production, replicate cultures were harvested after 4, 6, 8, and 10 days, and the potency of their enzyme extracts was compared. On approximately the tenth day sporulation occurred, so longer incubation periods were not investigated. The results are shown in Table II. Ten-day old cultures were used thereafter.

Enzyme Treatment and Extraction Conditions. In determining the influence of pH during enzyme treatment, 1.0-gram portions of soybean residue were incubated for 6 hours at 37° C. with 10 ml. of the enzyme adjusted to several pH's and saturated with toluene. Parallel tubes without enzyme were run at each pH, and after filtration micro-Kjeldahl analyses were run on the filtrates. From duplicate experiments the relative extractions (100 times the N extracted at each pH divided by the N extracted at pH 4.6) at pH's of 4.4, 4.6, 4.8, and 5.0 were 14, 100, 45, and 27%, respectively.

In 6-hour incubation periods at pH 4.6 at temperatures of 28°, 32°, 37°, and 44.5° C., 10 ml. of enzyme acting on 1.0 gram of soybean residue resulted in extraction of 5.5, 6.2, 7.0, and 5.0 mg. of nitrogen, respectively, over the amount extracted by nonenzymatic controls. Further studies were conducted at 37° C. and at pH 4.6.

In assessing the effect of enzyme concentration on subsequent extraction of nitrogen from soybean residue, 1.0-gram portions of soybean residue were incubated in the presence of toluene for 24 hours at pH 4.6 and 37° C. along with nonenzymatic controls (Table III).

Table II. Effect of Culture Age on Enzyme Potency[a]

Age of Culture, Days	Extraction of Nitrogen, %
4	26.3
6	27.9
8	29.2
10	29.6
Control (no enzyme)	21.4

[a] Here 10 ml. of enzyme from each culture were carried through the gel filtration and incubated with 1.0 gram of soybean residue for 6 hours at pH 4.6 in the presence of toluene. After filtration through Whatman No. 1 filter paper, micro-Kjeldahl analyses were made on the filtrates to determine the per cent of the total nitrogen which was extracted. Control (no enzyme) values have not been subtracted from the other values. The nitrogen contribution of the enzyme itself has also been subtracted.

Table III. Effect of Enzyme Concentration on Subsequent Extraction of Nitrogen from Soybean Residue[a]

Amount of Enzyme, Ml.	Soybean Residue Nitrogen Extracted, %
10	14
20	28
30	33
40	48
60	56
80	52
100	60

[a] After adding the appropriate volumes of enzyme (0.8 mg. of protein per ml.) to 1.0 gram of soybean residue, at pH = 4.6, all vessels were brought to the same volume by addition of water and saturated with toluene. Controls without enzyme were run in parallel and resulted in 6% extraction. Micro-Kjeldahl analyses of filtrates after 24 hours of incubation at 37° C. were then conducted. The values shown indicate the amount of nitrogen extracted in excess of that obtained in the control extract not treated with enzyme. Also subtracted prior to calculating the per cent extraction was the nitrogen contributed by the enzyme itself.

Analysis of filtrates from 1-gram portions of soybean residue incubated with 40 ml. (32 mg.) of enzyme at pH 4.6 and 37° C. in the presence of toluene for varying periods of time yielded the data shown in Table IV. Here aliquots of the filtrates were deproteinized in 10% trichloroacetic acid and analyzed for nitrogen after removal of the precipitate to permit estimation of the nonprotein nitrogen in the extracts (*2*). Progressive proteolysis was evident. Approximately the same percentages of nonprotein nitrogen were encountered in the nonenzymatic controls indicating that the protease activity is of soybean rather than *Pestalotiopsis* origin.

In assessing the nature of the polysaccharides that were being degraded concurrently with the release of protein, soybean residue was extracted with water both before and after enzyme treatment. Then the soluble sugar products were identified by paper chromatography and analyzed colorimetrically after elution of the spots from the paper chromatograms with the results shown in Table V.

Preparation of Fractions for Feeding Experiments. Two samples of 600 grams of soybeans each were soaked overnight in 2 liters of water for 12 hours. After the water was discarded, the beans were dehulled, homogenized in 5.1 liters of distilled water for 10 minutes in a Waring Blendor, and then filtered through two layers of cheesecloth. The filtrates were boiled for 1 hour to inactivate trypsin inhibitor and hemagglutinins and then lyophilized. The residue was lyophilized and treated with either distilled water or enzyme. Enzyme treatment was carried out at 37.5° C. and pH 4.6, using 32 mg. of enzyme protein per gram of dry residue in the presence of toluene. A control batch was treated in exactly the same manner except that no enzyme was used. After treatment, the filtrates and residues were filtered through Whatman No. 1 paper with suction, boiled for 1 hour, and lyophilized. The dried filtrates were combined with those obtained earlier in the fractionation. Material balance information and the proximate composition of the fractions appear in Table VI.

Table IV. Time Course of Enzymatic Modification of Nitrogen Extractability from Soybean Residue

Incubation Time, Hours	Extraction of Nitrogen from Soybean Residue, %	Nonprotein Nitrogen in Extract, %
2	33	42
4	40	55
7	49	61
24	65	69
48	65	80
72	65	81

Here 1.0-gram portions of soybean residue were incubated for varying times with 32 mg. of enzyme protein at pH 4.6 and 37° C. in the presence of toluene along with nonenzymatic controls. After filtration portions were analyzed directly and other portions after deproteinization in 10% trichloroacetic acid to determine extractability of nitrogen and the extent of proteolysis. Values obtained with the nonenzymatic controls have been subtracted, so that the values above indicate extractions in excess of the control values. Also subtracted prior to the calculation of these values was the nitrogen contributed by the enzyme itself.

Table V. Carbohydrate Reaction Products from Degradation of Soybean Residue

Carbohydrate	Source of Extract: Residue treated with boiled enzyme, mg.	Residue treated with active enzyme, mg.
Total	36	128
Reducing sugars (as glucose)	38	74
Glucose	3.8	10.0
Galactose	3.8	18.1
Arabinose	17.5	15.0
Xylose	2.5	2.0
"Disaccharide"	2.5	2.5

After incubation of 1.0 gram of soybean residue with 8 mg. of enzyme at pH 4.6 and 37° C. in the presence of toluene for 6 hours, the filtrates were analyzed for total sugars and degree of polymerization (*19*) and for reducing sugars (*17*). The sugars were also separated paper chromatographically (*21*), and the reducing sugars in each spot were determined after extraction of the paper with water. The "disaccharide" has not been identified.

The amino acid composition of soybean milk and residue prepared with and without enzymatic pretreatment and the amino acid composition of the additional protein extracted as a result of enzymatic treatment appear in Table VII.

Animal Evaluation of Protein Quality. Using the salt and vitamin mixtures of Campbell (*3*), diets containing 10% protein derived from the various soybean fractions or from casein were prepared as described by Derse (*4*). Groups of male Sprague-Dawley rats weighing 54 ± 5 grams each were fed each diet *ad libitum* in metabolism cages for 1 week. Daily food consumption and the 7-day weight change were recorded. Urine was collected in 1*N* HCl under toluene, and feces were collected daily and frozen. Carcasses were dissolved in hot 50% sulfuric acid, and aliquots were removed for nitrogen analyses. The results in terms of several parameters of protein quality appear in Table IX.

Table VI. Composition of Soybean Milk and Residue Produced with and without Enzymatic Pretreatment

Components	Control Process			Enzymatic Process		
	Milk	Residue	Total	Milk	Residue	Total
Dry matter, g.	305	183	488	393	79	472
Protein, g.	137	48	185	177	19	196
Crude fiber, g.	1	33	34	7	10	17
Proximate analysis, dry basis, %						
Protein	45	26		45	24	
Ash	6	1		6	3	
Ether extract	20	18		12	22	
Crude fiber	Nil	18		2	13	
Nitrogen-free extract	29	37		35	39	

Table VII. Amino Acid Composition of Enzymatic and Nonenzymatic Soybean Preparations (Grams per 100 grams of crude protein)

Amino Acid	Control Process		Enzymatic Process		Added Protein Attributable to Enzymatic Treatment
	Milk	Residue	Milk	Residue	
Lysine	7.3	8.0	8.0	8.7	9.9
Histidine	2.9	3.7	2.8	3.1	2.5
Arginine	6.4	6.2	4.8	3.5	0.2
Aspartic acid	10.6	11.5	10.3	10.3	9.5
Threonine	3.6	4.3	3.7	4.0	3.7
Serine	4.2	5.1	4.2	4.0	4.0
Glutamic acid	16.8	16.7	15.8	11.7	12.8
Proline	5.2	6.3	5.2	4.9	5.1
Glycine	3.9	4.6	3.9	5.0	4.0
Alanine	4.0	4.5	4.2	5.6	4.5
Cystine	2.7	1.4	2.4	4.0	1.6
Valine	4.3	5.4	4.5	5.5	5.3
Methionine	1.1	1.1	1.1	1.0	1.0
Isoleucine	4.3	5.4	4.2	4.6	4.1
Leucine	6.7	8.8	6.7	5.1	6.4
Tyrosine	3.4	2.5	3.2	2.7	2.7
Phenylalanine	4.4	5.3	4.3	4.5	3.7
Tryptophan	1.4	1.3	1.3	1.3	1.3
Ammonia	1.8	2.0	2.2	2.5	3.4

Table VIII. Capacity of Control and Enzyme-Treated Soybean Milk to Meet Essential Amino Acid Needs of Human Infants (*11*)

Amino Acid	Requirement, Mg. per Kg.	Satisfied by 2.6 Grams of Soy Milk Protein, %	Control
Histidine	34	215	220
Isoleucine	126	87	89
Leucine	150	116	116
Lysine	103	202	184
Methionine	45	64	62
Phenylalanine	90	124	152
Threonine	87	111	107
Tryptophan	22	154	163
Valine	105	111	107

Discussion

As a result of the enzymatic treatment of the initial residue, the amount of protein recovered in the milk has been raised from 74 to 96% (Table VI). This observation demonstrates the technical feasibility of the process. It was considered beyond the scope of the present study to explore the economic feasibility, since that requires careful study of means of optimizing both the production of enzyme and its use, as discussed later.

The data in Tables VI, VII, VIII, and IX confirm the observations of Hackler *et al.* (*6*) in that improvement in both the yield and the quality of the protein is apparent. The improvement in quality appears to involve both better digestibility and a superior amino acid pattern despite the fact that concentrations of the most limiting amino acids, methionine and cystine, are not elevated as a result of enzyme treatment.

At present most of the effective industrially available cellulases are produced in Japan and are derived from species of the genera *Trichoderma*, *Aspergillus*, and *Penicillium*. For specific purposes these enzymes show real promise. However, the spectrum of potential genera is considerably broader than the more conventional organisms (Table I). The extent to which other strains of these genera might be even more potent producers of enzyme deserves further investigation.

Although enzymes satisfactory for testing the desired

Table IX. **Evaluation of Nutritional Quality of Soy Milk and Soybean Residues Prepared with and without Enzymatic Treatment**

Observation	Dietary Protein Sources[a]					
	None	Casein	Control milk	Enzymatic milk	Control residue	Enzymatic residue
Number of animals	10	10	10	10	5	2
Weight gain, g.	−7.8	24.7	11.0	11.7	10.6	3.5
Urinary N, g.	0.059	0.094	0.163	0.177	0.154	0.230
Fecal N, g.	0.039	0.115	0.195	0.159	0.162	0.280
Carcass N, g.	1.18	1.82	1.53	1.60	1.54	1.46
N intake, g.	0.0	1.02	0.87	0.84	0.66	0.67
Protein digestibility, %	....	92	82	85	81	57
Biological value, %	....	96	85	83	82	68
Net protein utilization, %	....	62	40	49	48	41
Protein efficiency ratio	....	3.9[a]	2.0[c]	2.2[c]	2.5[b]	0.8[d]

[a] Like superscripts indicate values that are not significantly different from each other at the level of $P = 0.05$.

hypothesis were obtained here, optimization of enzyme production in terms of substrate and such environmental factors as pH, ionic strength, osmotic pressure, aeration rate, incubation time, and extraction procedure has only been crudely approximated.

Soybean protein is now used as an infant food primarily in two forms, as pasteurized soy milk and as a powdered soybean protein isolate. The proteolysis demonstrated in Table IV represents a serious problem in the case of preparing the powdered product because usually the protein is precipitated isoelectrically. More information is needed on the relative contributions of the soybeans themselves and of the *Pestalotiopsis* to total protease action before means of controlling proteolysis can be developed.

Precisely the mechanism behind the improvement in protein extraction is impossible to state. Culture filtrates of the type used here are enzymatically complex including, as a rule, a variety of carbohydrases, proteases, lipases, and even some oxidases. Which of these are the cause of the added extractability of protein and which merely occur concurrently remains uncertain. The data in Table VI, however, show clearly that approximately 50% of the crude fiber is solubilized, and the data in Table V show that large amounts of galactose are produced in addition to glucose. This latter observation confirms the presence of active hemicellulase and probably pectinase components.

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