Enzymatic Hydrolysis of Cottonseed Protein

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The relative activities of ten commercially available proteolytic enzymes were tested on cottonseed cake. Two microbial proteases and bromelin were most active. The activity of the two microbial proteases was then evaluated at different pH, temperature, enzyme, and substrate concentration values. One enzyme showed inactivation by substrate and both showed inactivation by gossypol. Between 40 to 60% protein solubilization was obtained under optimal experimental conditions.

The processing of oleagineous seeds results in two major products, vegetable oil and a leftover or cake. The cake is generally a good source of protein but its utilization either directly or indirectly in the human diet has been minor because of problems related with its assimilation, presence of toxic components, flavor, and texture. Cottonseed is mainly processed for its oil content under conditions where protein quality decreases (Rolz *et al.*, 1970). Research and development efforts at a pilot and at an industrial level have been and are actually carried out in order to find the proper methodology and processing conditions that will optimize oil recovery, protein quality, and toxic component levels (Rolz *et al.*, 1971b,c). Some results have been obtained for the alkaline extraction and concentration of the protein present in the cake (Rolz *et al.*, 1971c). Protein concentrates have the great advantage of being physically more suitable and adaptable in many solid or semi-solid food products and enriched beverages than the original raw materials. Results obtained with cottonseed cake point out that the protein extraction varies according to the type of oil-extracting process and also that, in all cases, it is not complete (Berardi *et al.*, 1969; Rolz *et al.*, 1971a). In order to increase the extraction it is possible to use proteolytic enzymes that will make protein soluble by degrading it, or hemicellulases and cellulases to release bound protein so that it may be extracted. This process has been tested with fish (Cheftel *et al.*, 1970; Hale, 1969; McBride *et al.*, 1961; Sen *et al.*, 1962; Sripathy *et al.*, 1964), peanut, and sesame cakes and four kinds of beans (Sreekantiah *et al.*, 1969), soybean meal (Abdo and King, 1967; Fujimaki *et al.*, 1968; Yasumatsu *et al.*, 1966), mung beans (Hang *et al.*, 1970b), coconut (Tamoda, 1964), beef muscle (Miyada and Tappel, 1956), renderers' meat scrap (Criswell *et al.*, 1964), and recently with pea beans (Hang *et al.*, 1970a). This paper presents the experimental results of the enzymatic solubilization of protein from defatted cottonseed cake.

MATERIALS AND METHODS

Substrate. A powdered cottonseed substrate was prepared from defatted cottonseed flour in order to have as much insoluble protein as possible in the following way. Fifteen pounds of cottonseed were slurried with an equal weight of distilled water and cooked at 100°C for 6 hr. The cooked slurry was filtered and the filter cake was extracted with distilled water for 1 hr. The wash water was removed by centrifugation and the solids were dried employing air at 60°C. The resulting solids were ball-milled. The chemical composition of the substrate is given in Table I. The protein insolubilization treatment was done in order to test the enzymes with a substrate which would resemble closely one whose soluble protein had been previously extracted.

Enzymes. All the proteolytic enzymes and the hemicellulase tested were used as commercially available. The enzymes used are listed in Table II. The bacterial proteases selected for further study were the ones with code BP and HT200.

Assay Procedure. The proteolytic activity of every enzyme was tested using the following procedure. Ten grams of cottonseed substrate and 90 ml of buffer were placed in a 250-ml flask. The pH was adjusted for every enzyme within its optimum range, as listed by the enzyme manufacturer. One milliliter of toluene was used as a bactericide. The desired enzyme quantity was added and a 5-hr incubation period at 45°C was started. The samples were shaken periodically, the reaction mixture was immediately filtered through filter paper (Whatman No. 4), and the residue was washed with distilled water until an approximate volume of 250 ml was completed. The filtrate was analyzed for total protein content (nitrogen × 6.25). Controls without enzyme were prepared using the same procedure. Enzyme relative activity was calculated as follows:

$$RA = \frac{HP - CP}{CP} \times 100$$

where RA = relative activity; HP = total protein obtained in the enzyme assay; and CP = total protein found in controls. The larger the difference between the protein solubilized by enzymic attack and that solubilized by the control, the higher the value of relative activity, calculated by the above formula. Controls were carried out for each enzyme tested.

Additional Experiments. With the best enzymes found in the above screening procedure, experiments were also done to study the effects on enzyme activity of enzyme and substrate concentrations, pH, temperature, reaction time, and different types of substrates. Also gel filtration experiments of the hydrolyzates were performed.

Chemical Analysis. Nitrogen was determined using the Biuret method (Gornall *et al.*, 1949) or Kjeldahl's macrotechnique (AOAC, 1965). Moisture, total and free gossypol were assayed by the AOCS official methods (AOCS, 1969). Soluble protein was measured by Lyman's method (Lyman *et al.*, 1953) using 0.03 *N* NaOH. The absolute enzyme activities were measured by the modified casein digestion methods of Keay and Wildi (1970), however, diluting the enzyme with water rather than in 0.1% calcium acetate. The hydrolysates were gel filtrated on a 1.27 × 25.2 cm column filled with polyacrylamide P-10, 100–200 mesh, (Bio-Rad Laboratories). The elution rate was 24 ml/hr and the buffer system used was 0.1 *M* sodium acetate at pH 5. The concentration of proteinaceous material in the fractions was

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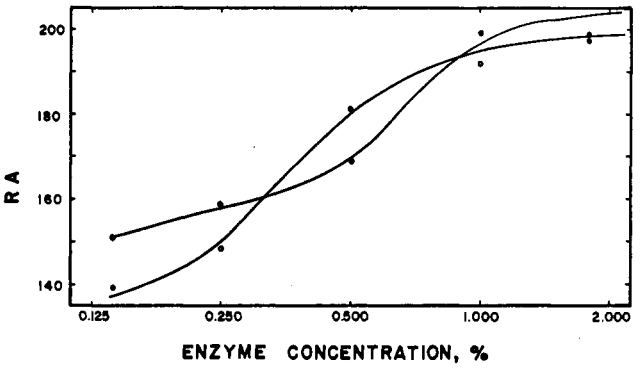

Figure 1. Effect of enzyme concentration, ○ BP, ● HT200

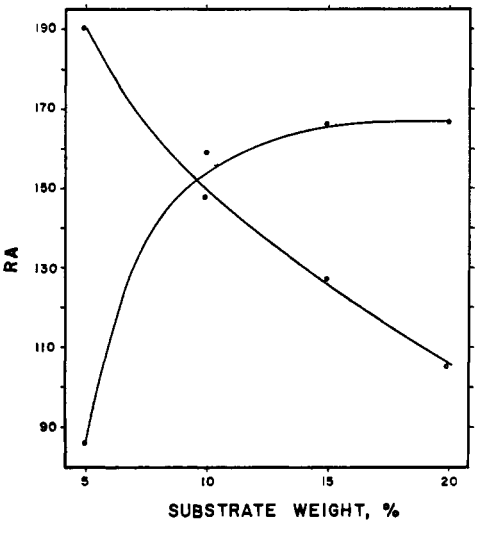

Figure 2. Effect of substrate concentration, ○ BP, ● HT200

Table I. Chemical Composition of the Cottonseed Substrate

	% in weight
Moisture	9.94
Total protein	45.65
Soluble protein	4.50
Residual oil	1.12
Total gossypol	0.996
Free gossypol	0.022

estimated by measuring the absorbance at 280 nm against the acetate buffer.

RESULTS AND DISCUSSION

In Table II the relative activities of the proteolytic enzymes on the cottonseed substrate are reported. For all the experiments a 0.25% concentration by weight of enzyme was used. As can be seen, in all cases there was a finite quantity of protein solubilized by enzymic action. The most active enzymes at these conditions were the two bacterial proteinases and the ones derived from higher plants (bromelin, papain, and ficin), which solubilized around 40% of the initial insoluble protein. The more activity shown by the bacterial proteinases on cottonseed substrate, however, cannot be generalized to another set of conditions or to some other substrates. For example, Sreekantiah *et al.* (1969), working with four different fungal proteases on defatted sesame meal, obtained good protein solubilization equivalent to relative activity values of 220 to 340. Also Criswell *et al.* (1964) and Connelly *et al.* (1966) found that the use of fungal proteolytic enzymes gave good results in separating protein from fat and bone. Hale (1969) tested 22 different enzymes on fish protein solubilization, and he found that a fungal protease from *S. griseus* showed the highest activity. However, one from *A. oryzae* was very low and below the values corresponding to proteolytic enzymes from higher animals, plants, and bacteria.

The activity shown by the hemicellulase was the lowest and it barely did facilitate the solubilization of nitrogenous compounds, contrary to what has been shown by Hang *et al.* (1970a,b) on mung and pea beans employing cellulase, where a large solubilization was noticed. Unfortunately no data were taken with a commercial cellulase and its effect on cottonseed substrate is unknown.

Effect of Enzyme Concentration. The effect of enzyme concentration on the relative activity of the two bacterial proteases (HT200 and BP) was studied. The results are presented in Figure 1. The amount of nitrogenous material solubilized as can be observed depends on the amount of enzyme added. With both enzymes the relative activity increases to a maximum of around 200 in the range of 1 to 2% of enzyme concentration. This means a 30% increase of RA for a 0.75% increase in enzyme weight. The same tendency as the one shown in Figure 1 has been reported by Sreekantiah *et al.* (1969) and Hang *et al.* (1970a,b).

Effect of Substrate Concentration. To establish the effect of substrate concentration on the solubilization of cottonseed protein, experiments were done varying the initial contents from 5 to 20%. The results are shown in Figure 2. With the HT200 the amount of nitrogen solubilized increased with the substrate concentration, reaching a limiting value at 13–14%. With the bacterial proteinase (BP), however, a decrease in activity was apparent as substrate increased. This behavior suggests an enzymatic inhibition by the substrate (Laidler, 1958). In order to test this hypothesis a series of assays were performed where the proteolytic activity on casein of the two enzymes was evaluated as a function of free gossypol added. The results are given in Table III. As can be seen, gossypol has a marked inhibitory effect on the activity of both enzymes, being greater with the BP where 85% inhibition was observed at 0.15% gossypol. This might explain its behavior shown in Figure 1. A similar reasoning, however, does not apply to the HT200, where, although there exists a 47% inhibition by gossypol (Table III) when tested with casein, its behavior, when varying substrate concentration, is as expected in normal enzyme kinetics. Neutral microbial proteases are inhibited by metal-chelating agents (Hagihara, 1956). Gossypol, besides being able to form readily metal complexes (Ramaswami and O'Connor, 1968) is also a polyphenolic compound which can inactivate enzymes by chemical blocking of the substrate (Loomis, 1969) or/and enzyme active sites (Laidler, 1958). This fact has been also reported by Meinke (1952) and by Lyman (1965). No final conclusions can be offered on this inhibitory effect, however, until more experimental data are obtained.

Effect of Time on Enzyme Action. The results are given in Figure 3. The relative activities increase with time for both enzymes. When time is doubled to 10 hr, up to 60% of the initial protein is solubilized, which is equivalent to 20% more than in the standard assay. The same tendency has been observed before, and small protein increases have occurred even at 24 hr digestion time (Fujimaki *et al.*, 1968;

Table II. Commercial Proteolytic Enzymes Tested and Relative Activity on the Cottonseed Substrate

Code	Enzyme	Manufacturer	Relative activity (RA)	pH
FC	Ficin concentrate	Enzyme Development Corp.	126.8	6.8[b]
BP	Bacterial proteinase	Enzyme Development Corp.	147.1	7.2[b]
PC	Papain concentrate	Enzyme Development Corp.	129.1	7.2[b]
CE-100	Fungi hemicellulase	Miles Laboratories	13.2	6.8[b]
HT200	Bacterial proteinase (from *B. subtilis*)	Miles Laboratories	158.1	6.8[b]
HT1000	Bacterial proteinase (from *B. subtilis*)	Miles Laboratories	49.5	6.8[b]
BC	Bromelin concentrate 1100 (from *B. subtilis*)	Miles Laboratories	159.8	4.6[a]
AFP	Acid fungal protease	Miles Laboratories	44.6	4.6[a]
P	Pepsin	Wilson Laboratories	37.1	2.0[c]
T	Trypsin	Wilson Laboratories	70.1	8.0[b]

[a] 0.2 *M* phthalate, NaOH buffer. [b] 0.2 *M* KH_2PO_4, NaOH buffer. [c] 0.2 *M* HCl, KCl buffer.

Hang *et al.*, 1970a,b) although Abdo and King (1967) reported for soybean a constant value after 24 hr.

Effect of pH on Enzyme Activity. There is a considerable variation in optimum pH values of proteolytic enzymes. This depends not only on the enzyme source itself, but also on the substrate being degraded (Tamoda, 1964). Several tests were planned at different pH values ranging from 1 to 11. The results are given in Figure 4. They show a sharp maximum for both enzymes around 9. The same tendency has been observed for the alkaline proteases of microbial origin (Keay and Wildi, 1970), although the maximum varies between 9 and 11. Fungal proteases on the other hand show a maximum activity region corresponding to a wide range of pH values, as shown to be on sesame meal from 2 to 5 (Sreekantiah *et al.*, 1969).

Effect of Temperature of Enzyme Activity. In order to find the activity–temperature profile, a series of assays was done between 0 and 80°C. The results are shown in Figure 5. The HT200 has an optimum activity near 60°C; the BC however is near 45°C.

Effect of Different Types of Substrates. The experiments reported before were done with a cottonseed substrate where 90% of the protein was insoluble, as shown in Table I. In order to find out the effect on the relative activity of the two microbial enzymes of the amount of insoluble protein present in the substrate, an experiment was done employing cottonseed cake without any treatment. The result is shown and compared with corresponding values for the standard sub-

Table III. Enzymatic Inhibition by Gossypol[a]

Enzyme	Gossypol, %[b]	Activity units[c]
HT200	0	218.0
	0.022	153.0
	0.150	115.4
BP	0	272.1
	0.022	124.0
	0.150	41.4

[a] The assay procedure was that of Keay and Wildi (1970). Each enzyme was tested at their optimum pH and temperature according to the manufacturers. [b] Gossypol was added to the casein as gossypol acetic (prepared by the Southern Regional Research Laboratory, USDA) and the mixture was mixed and incubated for 15 min at the testing temperature. [c] One activity unit is equal to 0.5 μg of tyrosine liberated in 10 min at the test conditions (Keay and Wildi, 1970).

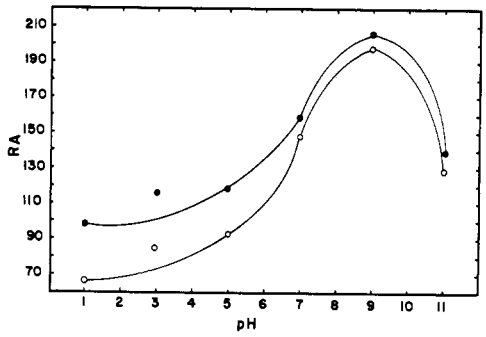

Figure 4. Effect of pH, ○ BP, ● HT200

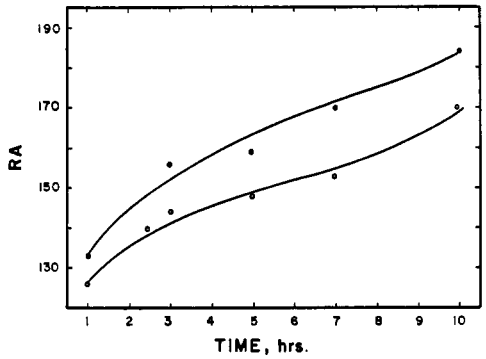

Figure 3. Effect of reaction time, ○ BP, ● HT200

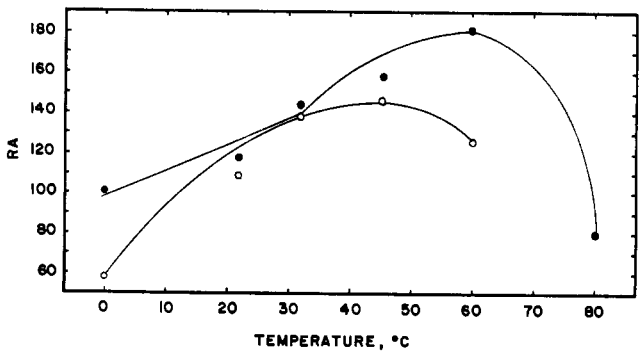

Figure 5. Effect of temperature, ○ BP, ● HT200

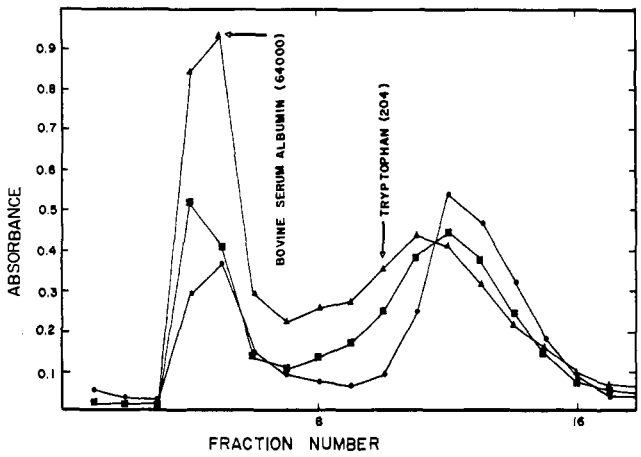

Figure 6. Enzyme HT200, gel filtration chromatography. Molecular weight of standards is shown in parentheses, ● pH 1, ■ pH 7, ▲ pH 11. Absorbance at 280 nm

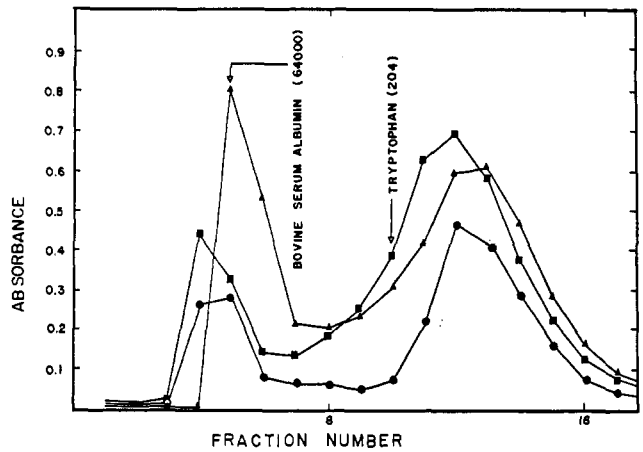

Figure 7. Enzyme BP, gel filtration chromatography. Molecular weight of standards is shown in parentheses, ● pH 1, ■ pH 7, ▲ pH 11. Absorbance at 280 nm

Table IV. Relative Activities for Two Substrate Types[a]

Substrate type	Enzyme	RA
Cottonseed cake as produced[b]	HT200	279.7
	BP	192.6
Treated cottonseed cake (results from Table II)	HT200	158.1
	BP	147.1

[a] Values obtained at pH 7.2, shows digestion time, temperature 45°C, 10% substrate concentration, and 0.25% enzyme concentration. [b] Cake from a pre-press solvent plant, with the following analysis: moisture = 11.64%; total protein = 39.00%; soluble protein = 30.72%; residual oil = 1.34%; total gossypol = 1.30%; free gossypol = 0.05%.

strate employed on this work in Table IV. The relative activities of both enzymes are greatly increased when cake as produced is used as a substrate (79% soluble protein). Sreekantiah *et al.* (1969) found the same general tendency with sesame, peanut, and bean meal. Earlier reports, however (Hoshino *et al.*, 1965, Tamura *et al.*, 1964), had found for soybean an increase of enzymic action with cooked (protein denaturated) substrates. It can be concluded, then, that the matter under discussion depends on the type of substrate and nature of the enzyme.

Gel Filtration of the Hydrolyzates. In order to determine the hydrolysis extent for both enzymes on the cottonseed protein, gel filtration of the hydrolyzates was carried out as a function of the hydrolysis pH values. The results are given in Figures 6 and 7. It can be seen that two peaks were always obtained, differing only in their height. On the figures are shown the elution fractions where some proteins, peptides, and amino acids run as standards where detected. The results suggest that the first peak corresponds to proteins of molecular weight around 64,000. This high molecular weight proteins are solubilized by buffers, as shown by Berardi *et al.* (1969). The second peak, as can be observed, corresponds to amino acids and low molecular weight peptides, which are the result of proteolysis. Cheftel *et al.* (1970) obtained a similar peak for their results on fish protein digestion. For both enzymes, an acid pH corresponds to a lower content of high molecular weight peptides.

With the results of the present study it is possible to select the most promising set of parameters for the enzymatic solubilization of cottonseed protein. The findings suggest that a one-step extraction procedure, employing a microbial enzyme active in the alkaline range, might prove to be the best method to extract most of the protein present in commercially available cottonseed cake. However, it would be desirable if more information is obtained with several other fungal proteases at low pH's where excellent results have been obtained with other substrates, and also to test the effect of cellulases on protein solubilization.

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Measurement of Tensile Strength of Muscle Fibers and Its Change during Postmortem Aging of Chicken Breast Muscle

Ryo Nakamura

To study the meat tenderization phenomenon, muscle fibers were prepared from the chicken pectoralis major muscle at a definite time after death and their tensile strength was measured. The tensile strength of muscle fibers increased a little until 6–8 hr postmortem and then decreased greatly. The tensile strength of muscle fibers prepared immediately after death, however, did not decrease during holding at 0°C for 24 hr and incubation with water extract of muscle did not decrease it. The cause of the changes in the tensile strength during postmortem aging is discussed.

Although many workers have been studying the meat tenderization phenomenon, its mechanism is not clarified yet. However, some interesting facts concerning the changes in meat during postmortem aging have been reported. One of these is the breaking of myofibrils into small fragments with mild homogenizing "fragmentation of myofibrils," which was first reported by Takahashi *et al.* (1967). Davey and Gilbert (1969) showed that morphological changes of myofibrils occurred in the region of the z-lines during storage and suggested that meat aging is due to disruption and possible dissolution of z-line material. If such morphological changes of myofibrils occur in stored meat, the tensile strength of muscle fibers can be expected to change during storage.

The tensile strength and the extensibility of muscle fibers were measured by some workers (Wang *et al.*, 1956; Sato *et al.*, 1967). However, no detailed studies have been made of the change in the tensile strength of muscle fibers during postmortem aging.

The major objectives of this study were to establish the measuring conditions of the tensile strength of muscle fibers and to investigate the changes in it during postmortem aging.

EXPERIMENTAL

Materials and Procedures. Twelve- to fourteen-month-old chickens of the White Leghorn (female) strain were used in these experiments. They were raised under similar environmental and nutritional conditions. The bird was slaughtered by cutting the jugular vein and carotid arteries, skinned without scalding, and eviscerated. The muscles were placed in a plastic bag without removing them from the carcass and stored in drained crushed ice.

Preparation of Muscle Fibers. Small pieces were cut from the pectoralis major muscle and were transferred into a petri dish containing a large volume of a "muscle preparative solution," such as 50% glycerol, 0.1 *M* KCl, 0.1 *M* KCl with 5 m*M* ATP, and Ringer's solution. (In this report, muscle fibers prepared in each solution were designated as 50% glycerol muscle fibers, 0.1 *M* KCl muscle fibers, and so on.) Then, small fiber bundles of uniform cross section were separated with two tweezers and transferred to another petri dish containing the same muscle preparative solution as the first, and their tensile strength was immediately measured.

The size of fiber bundle cross section was adjusted to that of about 25 single fibers, based on the following preliminary experiment. Muscle fiber bundles with various cross sections were prepared and the number of constituent single fibers was counted in each fiber bundle under a microscope. From this experiment, a definite relationship was noted between the size of the muscle fiber bundle and the number of constituent single fibers.

From the previous experiment using muscle fibers which were prepared in the preparative solution with different pH, it was noted that the tensile strength of muscle fibers was not affected by pH within the range 5.0 to 8.0. So, no buffer solution was used in this experiment for the preparation of muscle fibers.

Measurement of Tensile Strength. With scotch tape, 8–10 muscle fiber bundles longer than 2 cm were stuck on a small piece of cardboard (2 × 3 cm) which hung from the lever of

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