Rapid Analysis of Changes in the Molecular Weight Distribution of Buffer-soluble Proteins During Germination of Wheat[1,2]

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ABSTRACT

A high-performance liquid chromatography (HPLC) system equipped with Spherogel-TSK 3000 aqueous size-exclusion column and a computing integrator with a molecular weight program was used to monitor changes in buffer-soluble wheat protein components during germination of wheat kernels. At least seven major components were present. During germination, small molecular weight entities were progressively formed from proteins; some bind to the column and have longer column-elution times than their molecular weights would indicate. Such changes can indicate the approximate degree of germination of wheat kernels. Molecular weight profiles of extracts changed upon standing, indicating the presence of proteolytic enzymes. Cultivar differences in chromatographic elution profiles were very small.

Preharvest germination of wheat adversely affects bread-making quality, resulting in bread having a wet, sticky crumb. This effect is usually ascribed to the effect of increased α-amylase activity upon the starch component of dough (Buchanan and Nicholas 1979). Endogenous effects on other biochemical components, such as protein, may occur if sprout damage is extensive. Under certain conditions, germination may occur without visible evidence of sprouting; this is referred to as incipient sprouting. A rapid method is needed to assess whether such damage has occurred.

Over the last few years, high-performance aqueous gel permeation chromatography has been used increasingly for determining the molecular weight distribution of proteins (Fukano et al 1978, Kato et al 1980, Somack et al 1980). The major advantage of this technique is its rapidity. For example, the molecular weight of a protein can be determined in 30 min, as contrasted with at least 8 hr by conventional gel permeation chromatography. A further advantage of this technique is that very small volumes can be analyzed. The technique was recently applied to the study of cereal proteins (Bietz and Cobb 1981), but its application to the analyses of proteins in germinating wheat has not been investigated.

The present study was performed, therefore, with three objectives in mind: to determine the potential of high-performance aqueous gel permeation chromatography in determining the molecular weight profiles of buffer-soluble proteins present in sound and germinated wheat kernels; to determine whether changes in the chromatographic profiles of buffer-soluble wheat proteins could be used as indicators of the stage of germination; and to determine whether changes in the molecular weight profile of extracted proteins with time can be used as indicators of proteolytic activity.

MATERIALS AND METHODS

Wheat Samples

Selected for this study were the hard red spring wheat cultivars Glenlea and RL 4132 (unlicensed), the soft white spring cultivar, Idaed, and the durum wheat cultivar, Wascana.

Germination of Wheat Samples

Wheat kernels were steeped for 2 hr in distilled water at 22°C, spread evenly on moist blotting paper, and germinated at 18°C for one to six days in a germination cabinet at 100% humidity. The samples were then air-dried on a bench top at 22°C. The final moisture of the wheat kernels was 10.5%.

Extraction of Buffer-soluble Proteins

A Udy cyclone mill with an 18-mesh screen (Udy Corp., Boulder, CO) was used to finely grind wheat kernels. One gram was stirred at room temperature with 10 ml of 0.05 *M* sodium phosphate buffer, pH 7.0, containing 0.5*M* sodium chloride, for 10 or 60 min, and filtered through a Millipore aqueous-sample clarification kit with a 0.45 μm filter. The extract was immediately injected onto the high-pressure liquid chromatograph (HPLC) for analysis of buffer-soluble proteins. Part of each extract was stirred for an additional 2 hr before analysis to assess possible changes caused by proteolytic activity.

High-Performance Aqueous Gel Permeation Chromatography

A model ALC/GPC HPLC (Waters Associates, Milford, MA) consisting of a Wisp Automatic-Sample Injector, a model 6000A pump, and a model 440 absorbance detector (280-nm filter) and attached to a Spectrophysics SP4000 computing integrator (Technical Marketing Assoc., Mississauga, Ontario) with digital interface and printer plotter was used. The column selected for analyses of the molecular weight distribution of proteins was a 30 × 0.75-cm Spherogel-TSK 3000 column (Altex Scientific, Berkeley, CA), preceded by a 7.5 × 0.75-cm Spherogel-TSK 2000 precolumn. The buffer used was 0.05 sodium-phosphate buffer, pH 7.0, containing 0.5*M* sodium chloride. The flow rate was 1 ml/min. The exclusion limit for proteins was approximately 500,000 daltons (Kato et al 1980). $V_o + V_T$ for the column were 7.2 and 20.1 ml, respectively. The column was calibrated with a gel-filtration standard from Bio-Rad Laboratories (Richmond, CA) and consisted of thyroglobulin (6.7×10^5 daltons), γ-globulin (1.58×10^5 daltons), ovalbumin (4.4×10^4 daltons), myoglobulin (1.7×10^4 daltons), and vitamin B-12 (1.35×10^3 daltons). A program supplied for the computing integrator was used to give the molecular weight distribution. Injection volumes were 50 or 100 μl.

RESULTS AND DISCUSSION

The same extractant and eluent buffers were used in this study to simplify the method and make it as rapid as possible, and to avoid possible precipitation of protein on the HPLC column. The buffer, 0.05*M* phosphate, pH 7.0, containing 0.5*M* sodium chloride, was chosen to minimize adsorption of protein onto the slightly hydrophilic Spherogel-TSK columns. For example, when nonphosphate buffers are used at other than near neutral pH, such as acetate buffer, pH 5.0 or Tris-HCl, pH 8.6, protein recovery (Altex Scientific Inc., private communication) is affected. Similarly, at least 0.2*M* sodium chloride was required to obtain high recovery of proteins having high pI. Using this extractant, albumin and globulin proteins, as well as low molecular weight

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peptides and amino acids, would be extracted, but gluten proteins would remain largely insoluble.

Changes in the molecular weight distributions of buffer-soluble proteins, peptides, and amino acids in sound kernels and in kernels germinated from one to six days (hard red spring wheat Glenlea and durum wheat Wascana) are shown in Fig. 1. Results are presented for 10 and 60 min of extraction and for centrifuged extracts (10 min) stirred for an additional 2 hr. It can be noted that HPLC analysis was complete in 32 min, after which no further components eluted.

For simplicity, the chromatograms can be divided into four molecular weight groups. High molecular weight proteins elute at the void volume of the column (6–8 min elution); intermediate molecular weight proteins, of approximately 4,000–100,000 daltons, elute at 8–16 min; low molecular weight peptides and amino acids elute after the lower exclusion limit of the column (16–22 min); and components that adsorb to the column elute later than their molecular weights would indicate (22–32 min).

From Fig. 1 it is evident that intermediate and high molecular weight proteins do not change dramatically during germination. The largest changes are in the progressive buildup of low molecular weight peptides and amino acids eluting after V_T. This confirms previous findings that increases in solubilized amino nitrogen upon germination are due predominantly to amino acids (Preston et al 1978). Such amino acids are formed by two nonspecific carboxypeptidases and two aminopeptidases that are present in the endosperms of mature and germinating wheat (Preston and Kruger 1976,1977; Kruger and Preston 1977,1978). Although an endoprotease also forms progressively upon germination, the peptidases are able to rapidly hydrolyze the peptides produced to amino acids (Preston et al 1978).

Three components eluting at approximately 23, 27, and 31 min are apparently highly charged and adsorb to the column. Because monitoring in this study was done at 280 nm, one cannot state unequivocally that the components are proteinaceous in nature. The latter two components form only upon germination and could be potential markers of the extent of sprout damage in wheat samples. Unfortunately, the amounts of such components are small in comparison to the main components.

To determine whether a 10-min extraction time was sufficient to solubilize buffer-soluble protein, a 60-min extraction was also performed. As shown in Fig. 1, changes in protein patterns were slight. So that changes could be assessed quantitatively, chromatograms were divided into seven parts representing the major components of the chromatogram (Fig. 2). Areas were computed using the computing integrator for the 10- and 60-min extraction times of zero- and six-day extracts of Glenlea wheat, and compared as a percentage. As shown in Table I, there was a slight decrease in the high molecular weight component and a maximum increase of 37% for the other components, presumably because of a

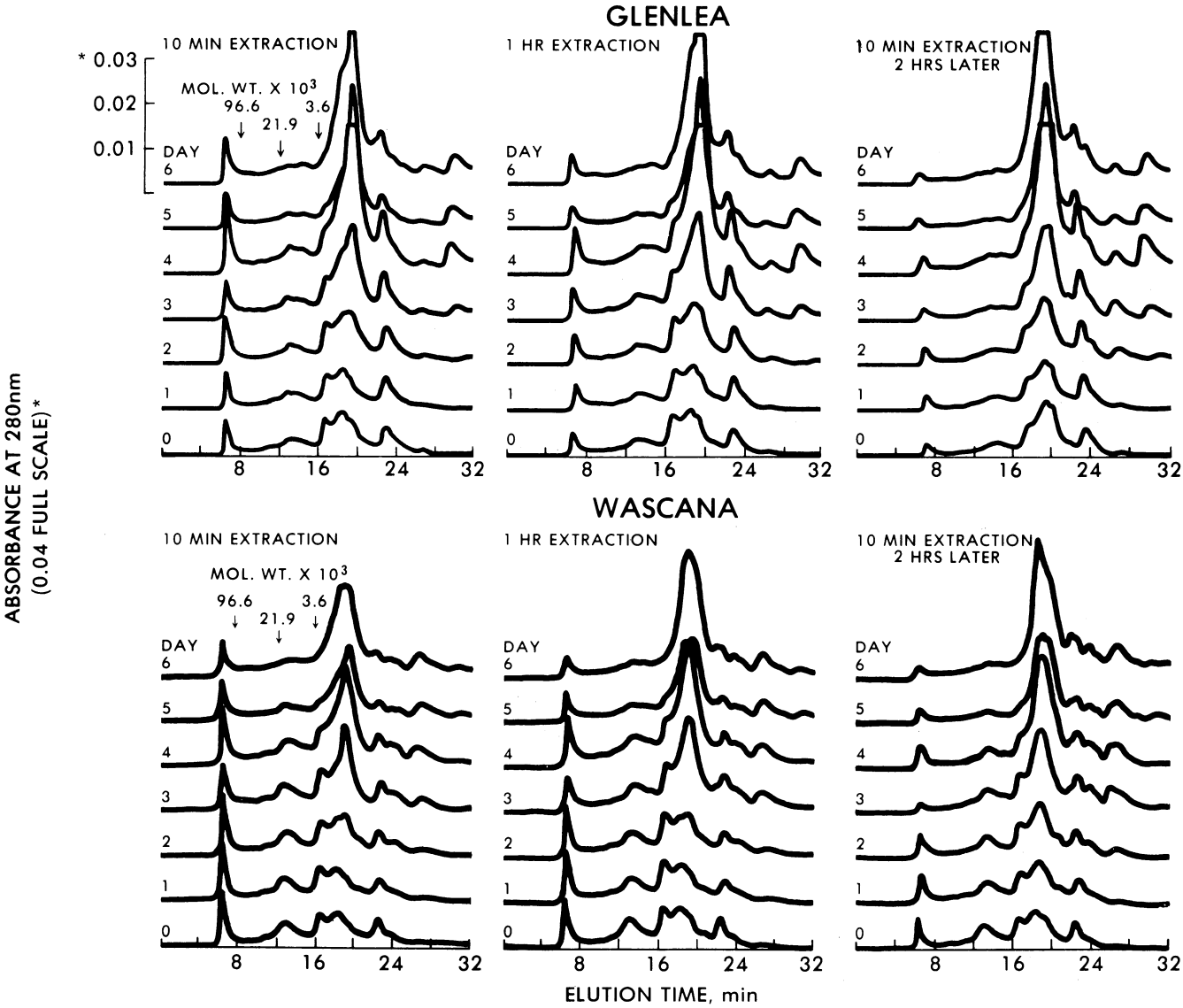

Fig. 1. Molecular weight distribution of buffer-soluble proteins in Glenlea and Wascana ungerminated and germinated wheat. **Left,** 10-min extraction; **middle,** 1-hr extraction; **right,** 2-hr stirring of 10-min extract. Injection volumes were 100 μl for wheat germinated for zero and four days and 50 μl for wheat germinated for five and six days.

more complete extraction. Ungerminated and six-day germinated wheat extracts behaved similarly. This indicated that the increased endoproteolytic activity present in six-day germinated wheat causes minimal degradation and added solubilization of peptide fragments under such extraction conditions. Substantial changes occurred, however, in the chromatographic profile of 10-min extracts that were allowed to stir for 2 hr after filtering (Fig. 1). The percentage of change in areas for the major chromatographic components from extracts of ungerminated and six-day germinated wheat are indicated in Table II. The major change was a large decrease in component 1 (the high molecular weight components) and substantial increases in lower molecular weight components. The decrease in high molecular weight components was more pronounced with six-day germinated wheat extract, and decreases were also present in intermediate molecular weight components (4,000–100,000 daltons). These changes are probably due to carboxypeptidases in the extracts (Preston and Kruger 1976), particularly because the decreases in high molecular weight proteins are accompanied by corresponding increases in very low molecular weight species. That such carboxypeptidases increase approximately twofold upon germination explains the larger effect observed in the germinated wheat extract. These findings emphasize the importance of analyzing buffer-soluble wheat proteins as soon as possible after extraction.

The hard red spring wheat Glenlea and the durum wheat Wascana had very similar chromatographic profiles for buffer-soluble proteins (Fig. 1). The main difference between the two cultivars was in the adsorbed peaks eluting at 27 and 31 min. With the hard red spring wheat, the component eluting at 31 min increased upon germination, whereas with the durum wheat the component eluting at 27 min was the major adsorbed component to increase. The hard red spring wheat RL 4137 and the soft white spring wheat Idaed were also very similar in chromatographic profiles to the Glenlea and Wascana wheat when extracts from ungerminated wheat were compared (Fig. 3). Germinated wheat extracts of RL 4137 and Idaed, however, were similar to Glenlea but not Wascana, since they possessed the adsorbed component eluting at 31 min rather than the component eluting at 27 min. This may be attributed to the genetic differences between hexaploid and hetraploid wheats.

Of the major components in Fig. 2, component 4, eluting between 19 and 22 min, appeared most promising as a potential indicator of the stage of wheat germination because of the large increases in amounts of this component. Late-eluting components could also be used, but increases were much smaller. Increases in the area of this component upon germination were compared to the area of ungerminated kernels for the four varieties. The results in terms of percent increase in areas is shown in Fig. 4. Increases were similar for all cultivars: an increase of approximately six times occurred over six days of germination. By comparison to sound kernels, the method can thus roughly indicate the extent of germination.

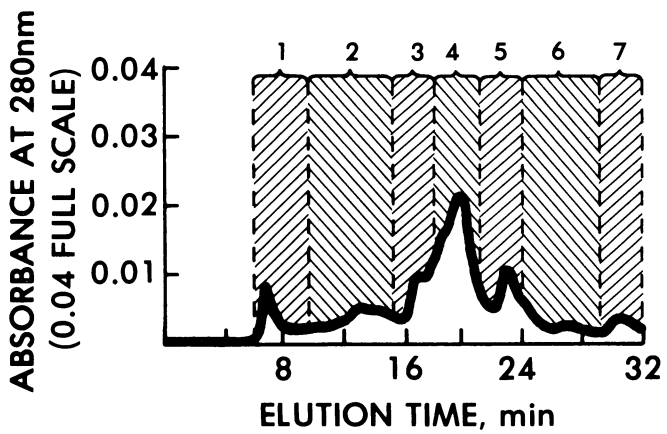

Fig. 2. Major components present in chromatograms of germinating wheat extracts.

DISCUSSION AND CONCLUSIONS

High-performance aqueous gel-permeation chromatography can rapidly determine the molecular weight distribution of buffer-soluble proteins in sound and germinated wheat kernels. If a 10-min extraction time were used, a complete analysis could be done within an hour. Because small volumes (50–100 μl) are required for analysis, single kernels can also be analyzed. Extracts should be analyzed promptly to avoid spurious changes in molecular weight distributions.

At least seven major components were present in extracts. Some components, however, adsorb on to the column even with very strong salt in the eluent. Consequently, representative molecular weights cannot be determined for such species. Such components increase upon germination, however, and could indicate sprouting in wheat samples.

All hexaploid wheats explained had very similar chromatographic profiles for the buffer-soluble proteins from both sound and germinated wheat kernels. As such, the present method would not be useful for cultivar identification within a class, although it may be possible to distinguish hexaploid from tetraploid wheats. Preliminary results with low-ionic-strength buffers, however, give separations that are not based on molecular weight but that nevertheless are potentially useful in separating cereal proteins. In addition, high-performance aqueous gel permeation chromatography of wheat gluten shows great promise for comparison of wheat cultivars, provided appropriate buffers for extractant and eluent are used (Bietz and Cobb 1981).

The major change in molecular weight distribution of the buffer-soluble proteins upon germination of wheat kernels was the formation of low molecular weight species. This finding is in agreement with previous work indicating that amino acids increase progressively during germination. The area of the low molecular weight component increases linearly with germination time, and this can be used as a guide to the stage of wheat kernel germination. Other experimental conditions may, of course, affect observed amounts of this component. Since very little change was observed during the first two days of germination, the method is unsuitable for detecting incipient sprouting, in which sprouting is not evident, although levels of enzymes have increased. Analyses of buffer-insoluble protein components are planned to determine whether sprouting can be detected earlier.

Analysis of proteins in germinating wheat kernels is an

TABLE I
Percent Change in Area of Components from Glenlea Wheat After 60 Min of Extraction Compared to 10 Min of Extraction Time

	Days of Germination	
Component and Time (min)	0	6
1 (7–10)	−1	−5
2 (10–16)	+37	+31
3 (16–19)	+32	+14
4 (19–22)	+17	+12
5 (22–25)	+17	+16
6 (25–30)	+14	+12
7 (30–33)	+25	+17

TABLE II
Percentage Change in Areas of Chromatographic Components of Extracted Proteins (10 min) from Glenlea Wheat After 2 Hr of Stirring

	Days of Germination	
Component and Time (min)	0	6
1 (7–10)	−39	−64
2 (10–16)	+10	−31
3 (16–19)	+11	−16
4 (19–22)	+33	+15
5 (22–25)	+32	+19
6 (25–30)	+95	+6
7 (30–33)	...	−2

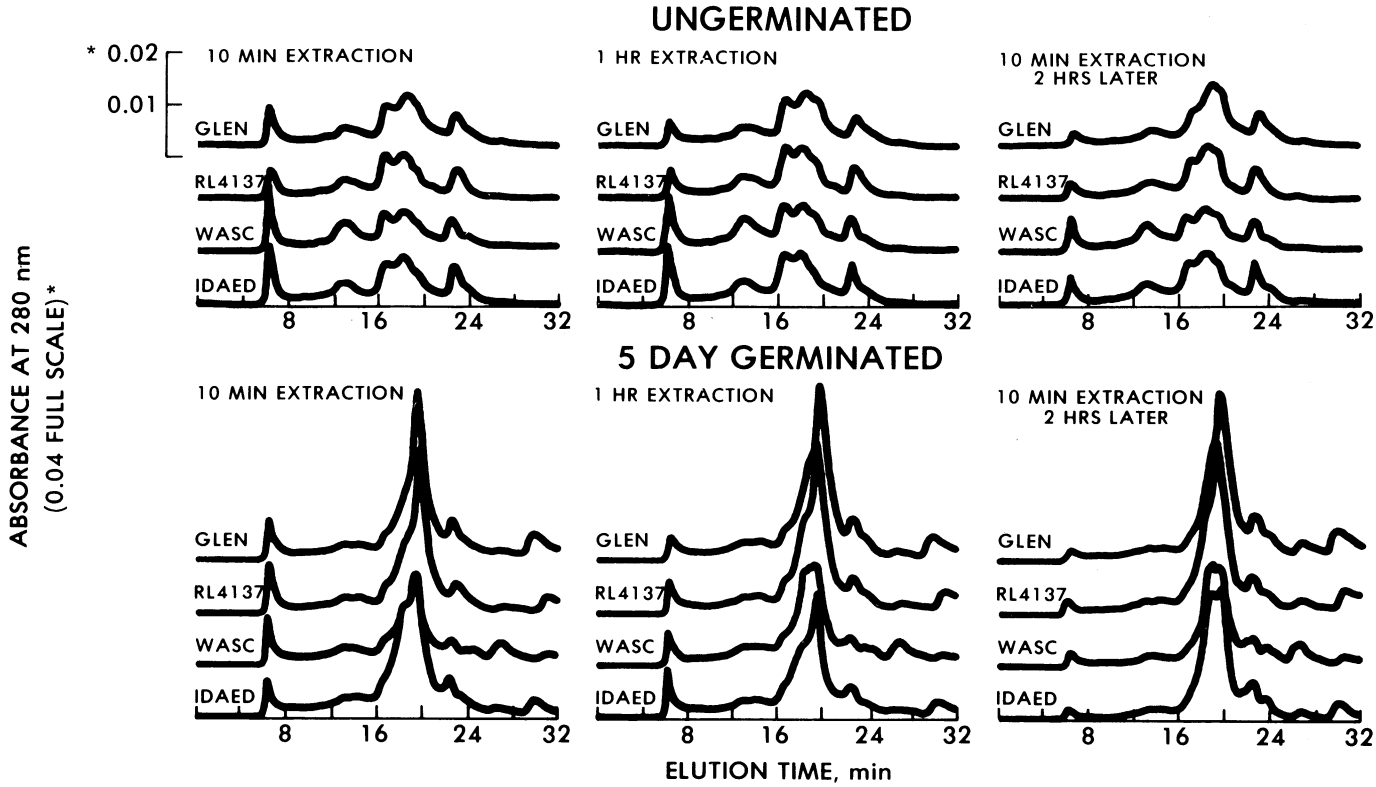

Fig. 3. Comparison of the molecular weight distribution of buffer-soluble proteins from Glenlea, RL 4137, Wascana and Idaed ungerminated and five-day germinated wheat extracts. **Left,** 10-min extraction; **middle,** 1-hr extraction; **right,** 2-hr stirring of 10-min extract. Injection volume was 100 μl for the ungerminated wheat and 50 μl for five-day germinated wheat.

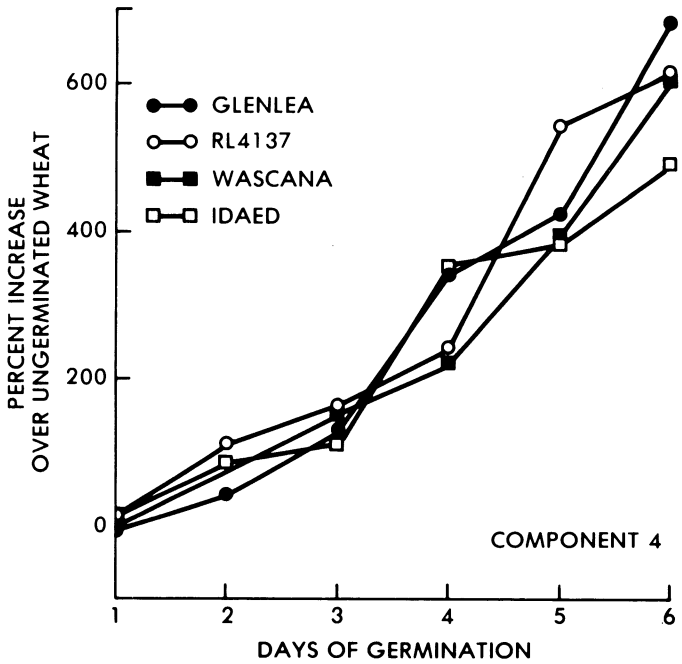

Fig. 4. Percent increase in the amount of component 4 (Fig. 2) upon germination.

indication of in vivo proteolytic degradation. Monitoring of extracts with time, however, does not reflect amounts of proteolytic enzymes present or their mode of attack under in vitro conditions. Such examination would be possible, however, if the extraction conditions were closer to the pH optima of such enzymes and if products of proteolysis were soluble in chromatographic eluent.

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