NOTE ON SEPARATION AND PARTIAL PURIFICATION OF WHEAT PROTEASES BY AFFINITY CHROMATOGRAPHY[1]

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Affinity chromatography on hemoglobin-substituted Sepharose has been shown to be potentially useful in the partial purification of plant proteases (1–3). Recent studies in our laboratory have shown that in addition to hemoglobin, a number of amino acid and dipeptide ligands coupled to Sepharose were effective for the partial purification of wheat proteases. Of the ligands tested, phenylalanine gave the largest increases in specific activity. Furthermore, elution of activity with salt gradients rather than with single high-salt buffers separated activity into a number of peaks and significantly increased specific activities. This note presents details of these studies.

MATERIALS AND METHODS

Amino acids and dipeptides were obtained from Schwarz-Mann, bovine serum albumin (fraction V) from Baker, hemoglobin from Worthington, cyanogen bromide from Aldrich, and Sepharose 4B from Pharmacia. All other chemicals were of reagent grade.

Preparation of Affinity Columns

The following describes the procedure for phenylalanine-substituted Sepharose. Concentrations used for other ligands are listed in Table I. Phenylalanine was coupled to Sepharose by the method of Cuatrecasas et al (4). A 65-ml suspension of Sepharose 4B was mixed with an equal volume of water, and 6.25 g of cyanogen bromide dissolved in 65 ml of water was added. The pH was immediately adjusted to pH 11.0 with 4*N* sodium hydroxide. The gel was stirred gently for 10 min and washed exhaustively with cold 0.1*N* sodium bicarbonate (pH 9.0); 60 ml of a 0.2% (w/v) aqueous solution of phenylalanine was added. The solution was stirred gently at 4° C overnight, washed with water, and equilibrated with buffer.

Preparation of Enzyme Extracts

Samples of ungerminated and five-day freeze-dried (18.5° C) germinated wheat (*Triticum aestivum* L. cv. Manitou) were ground in a coffee grinder (Krups model No. 50) and stirred for 1 hr at 4° C with 0.025*M* sodium acetate buffer, pH 4.0 (1:5 w/v). Extracts were then centrifuged 15 min at 35,000 × *g* (4° C) and the supernatant stored at 4° C until further use.

Affinity Chromatography of Proteases

Columns (22 × 1.7 cm) were prepared (4° C) by pouring a suspension of phenylalanine-substituted Sepharose and pumping through dilute buffer

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(0.025*M* sodium acetate pH 4.0). Enzyme extracts (15 ml) were then layered onto the gel and eluted with approximately 200 ml (30 ml/hr) of the same buffer. Activity was eluted by pumping through either 0.25*N* sodium acetate, pH 4.0 (15 ml/hr), or a convex salt gradient (15 ml/hr) produced in a Technicon Autograd gradient maker with the following buffers: 75 ml of 0.05*M* sodium acetate (pH 4.0), 75 ml of 0.20*M* sodium acetate (pH 4.0), and 75 ml of 0.20*M* sodium acetate (pH 4.0). Fractions (8 ml) were collected and analyzed for protease activity and protein.

Protease Activity Determination

Exoproteolytic activity was measured by an automated fluorometric assay using hemoglobin as substrate at pH 4.0 (5). Activity was expressed in terms of the equivalent fluorescence of glycyl-glycine (μmole/ml) following reaction of dialyzed products with fluorescamine.

Endoproteolytic activity was measured by the release of colored products (OD_{440}) from azocasein at pH 6.0 by the method of Kruger (6) except that samples were diluted 1:1 with substrate buffer before analysis and incubated 3.5 hr. Azocaseinase activity was expressed as the change in absorbance at 440 nm under the conditions of the assay.

Protein was determined by the method of Lowry et al (7).

RESULTS AND DISCUSSION

Initial studies of the effects of various amino acid, peptide, and protein ligands coupled to cyanogen bromide-activated Sepharose 4B were done with germinated wheat extracts using a two-step elution procedure. All columns tested gave optimum increases in specific activity of proteolytic enzymes at approximately pH 4.0. Results shown in Table I indicate that ligands containing hydrophobic amino acids (L-phenylalanine, L-proline, glycyl-L-proline) gave

TABLE I
Effects of Ligands on Affinity Chromatography of Germinated Wheat Proteases

Ligand	Ligand Concentration (%)	Hbase[a] Specific Activity[b]	Hbase Recovery (%)	Azocaseinase Specific Activity[c]	Azocaseinase Recovery (%)
Hemoglobin	4.0	12.6	83	.181	68
Bovine serum albumin (fraction V)	4.0	12.9	78	...	...
α-L-glutanyl-glutamic acid	0.4	11.9	85	...	...
L-phenylalanine	0.2	15.9	83	.217	72
L-proline	0.2	13.8	84	.184	71
L-lysine	0.2	9.2	80	...	...
L-glutamic acid	0.2	10.5	80	...	...
Extract		2.4	100	.048	100

[a]Hbase = hemoglobin-degrading enzymes.
[b]μmol of glycyl-glycine/mg of protein.
[c]ΔOD_{440}/mg of protein.

the largest increases in specific activity of hemoglobin-degrading enzymes (Hbase) as measured by an automated fluorometric assay (5), with phenylalanine being the most effective (Fig. 1). Protein ligands (hemoglobin, bovine serum albumin) gave smaller increases in specific activity, while ligands containing glutamic acid or lysine were least effective. Of the ligands tested for azocaseinase activity, phenylalanine again gave the largest increases in specific activity (Table I, Fig. 1). The low recoveries of azocaseinase activity from the columns are probably associated with its tendency to become unstable at higher purification levels.[2]

Similar results were obtained with ungerminated wheat extracts. Elution of activity from phenylalanine-substituted Sephrose gave the largest increases in specific activities of Hbase and azocaseinase (data not shown). Increases in specific activity, however, were significantly lower with ungerminated (2.5-fold increase) than with germinated wheat extracts. Recoveries of azocaseinase activity were slightly lower than were those obtained with germinated extracts. In contrast, recoveries of Hbase activity were approximately 160%. The increased recovery of Hbase activity from ungerminated wheat extracts probably was associated with the removal of endogenous inhibitors that were previously reported in extracts of maturing wheat (8).

[2]J. Kruger. Unpublished data.

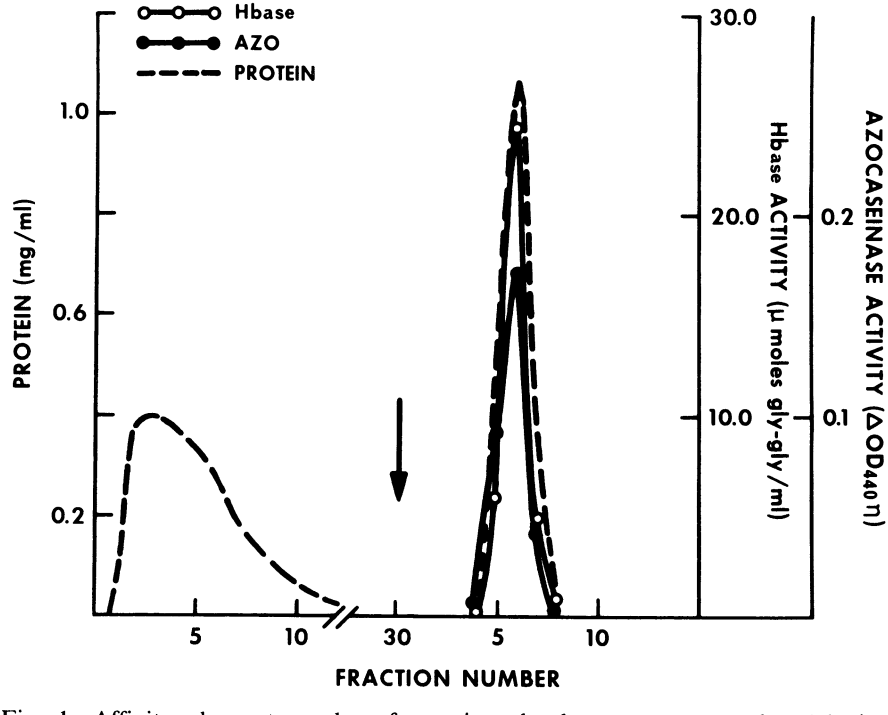

Fig. 1. Affinity chromatography of germinated wheat extract on phenylalanine-substituted Sepharose 4B. Proteolytic activity was eluted with 0.25*M* sodium acetate, pH 4.0.

The use of phenylalanine rather than hemoglobin-substituted Sepharose 4B was an improved method of affinity chromatography of wheat proteases, but the relatively small increases in specific activity, especially with ungerminated wheat extracts, suggested that nonspecific binding of large amounts of inactive protein occurred. When lower salt concentration buffers were used to elute activity from phenylalanine-Sepharose, however, the protein peak was eluted slightly before the activity peak. This suggested that the bulk of the absorbed nonactive protein had lower affinities than did the enzymes.

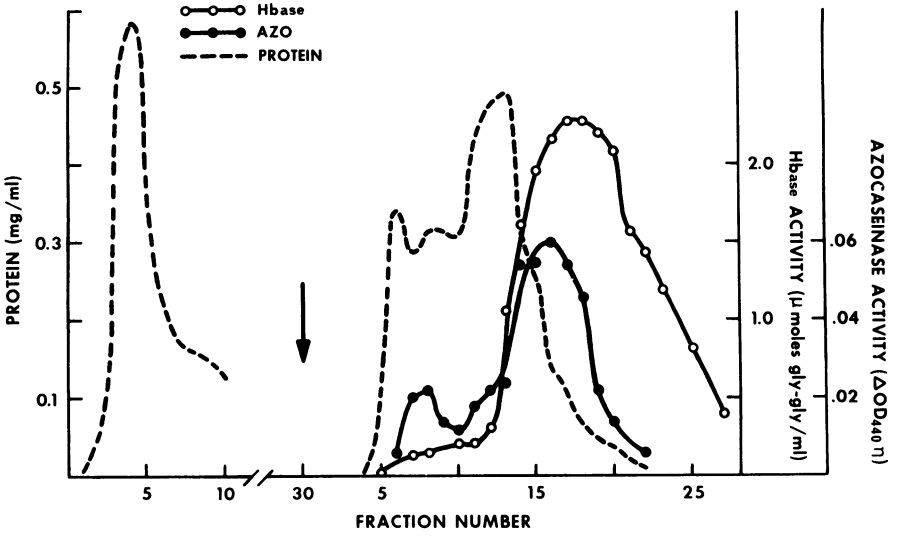

Fig. 2. Affinity chromatography of germinated wheat extract on phenylalanine-substituted Sepharose 4B using convex salt gradient for elution.

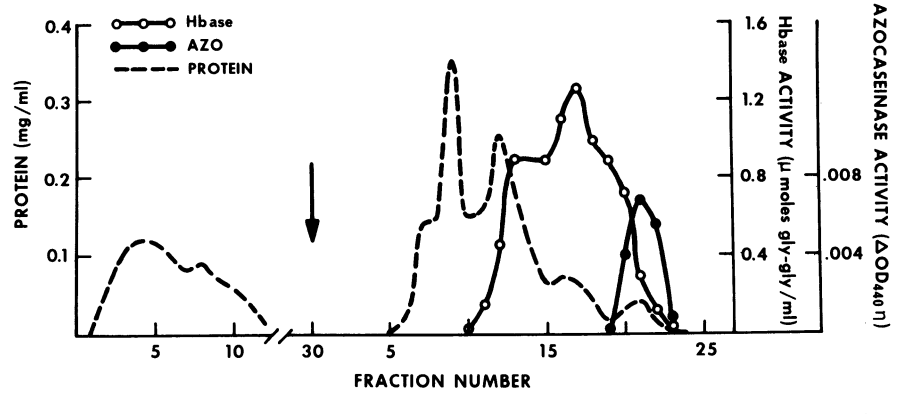

Fig. 3. Affinity chromatography of ungerminated wheat extract on phenylalanine-substituted Sepharose 4B using convex salt gradient for elution.

To separate nonactive protein from activity, elution with a variety of salt gradients was tested. The most efficient was elution with a three-buffer (0.05-0.20-0.20*M* acetate, pH 4.0) convex gradient. Figure 2 shows the resulting chromatography profiles for a germinated wheat extract using this procedure. Two azocaseinase and one broad Hbase peak were evident. Except for the minor azocaseinase peak, the proteolytic enzymes were effectively separated from inactive protein. Hbase activity showed an approximately 40-fold increase in specific activity compared with the crude extract, while the major azocaseinase peak increased 15-fold in specific activity. Recovery of Hbase activity from the column was approximately 75%, while total azocaseinase activity recovery was approximately 55%.

Figure 3 shows results with ungerminated wheat extracts. One broad Hbase and one azocaseinase peak were evident with specific activity increases of approximately 20-fold (130% recovery) and 12-fold (40% recovery), respectively. The elution position of the Hbase peak was similar to that found in germinated wheat extracts, but the azocaseinase peak appeared to be eluted at higher salt concentration than were either of the azocaseinase peaks in germinated wheat. These results are consistent with recent studies in our laboratory that indicate that in contrast to exoproteases (Hbase), which show little change in properties during germination, endoproteases (azocaseinase), which cause increases in activity during germination and appear to be responsible for the control of storage protein hydrolysis, have properties different from those present in ungerminated wheat (9).

The present results indicate that affinity chromatography on phenylalanine-Sepharose columns using salt gradient elution may be useful in the purification of wheat proteases. In addition to obtaining substantial increases in specific activity, the method can separate enzymatic activity into several fractions, presumably on the basis of varying affinities of the enzymes. The present study was restricted to wheat, but perhaps the method also can be applied to the partial

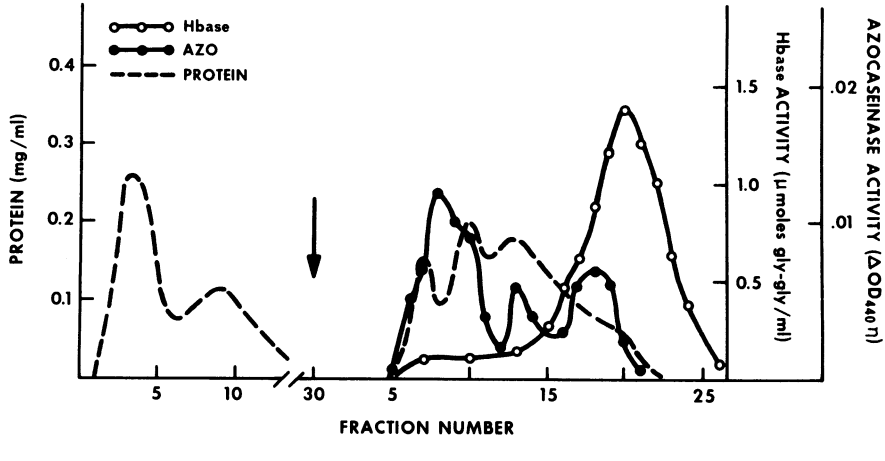

Fig. 4. Affinity chromatography of malted barley extract on phenylalanine-substituted Sepharose 4B using convex salt gradient for elution.

purification and separation of other plant proteases. For example, malted barley extracts can be separated into one Hbase and three azocaseinase peaks (Fig. 4) with significant increases in specific activity, while germinated corn proteases[3] yield at two proteolytically active peaks.

[3]K. Preston and A. Oaks. Unpublished data.

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