PURIFICATION OF WHEAT PROTEASES BY AFFINITY CHROMATOGRAPHY ON HEMOGLOBIN-SEPHAROSE COLUMN[1]

G. K. Chua and W. Bushuk

Protein Chemistry Laboratory, Department of Plant Science, University of Manitoba, Winnipeg 19, Manitoba, Canada.

Received September 15, 1969

SUMMARY

A rapid chromatographic procedure for the isolation and purification of proteases from malted wheat flour is described. The separation was achieved by passing the crude extract through a hemoglobin-Sepharose column. Unadsorbed proteins were eluted with the starting buffer. The adsorbed proteases were then eluted with 0.1N acetic acid. Recoveries of proteins and proteolytic activity were over 90%. A two-fold increase in specific activity was achieved by this purification technique. Disc electrophoretic analyses showed that all of the non-proteolytic components were separated from the proteolytically active proteins. The active peak comprised three major and one minor proteins of similar mobility.

Difficulties encountered in the isolation and purification of wheat proteases have prevented the fundamental characterization of these technologically important enzymes. The main difficulty appears to be due to the very low proteolytic activity of wheat (McDonald, 1969) and the tendency of its proteases to aggregate with other proteins (Wang and Grant, 1968; Chua and Bushuk, 1969).

Recently Cuatrecasas et al. (1968) have purified several enzymes by first adsorbing them on Sepharose containing covalently bound substrate analogues. Our communication will describe an analogous chromatographic procedure which was used successfully in the purification of wheat proteases. The insoluble support material was also Sepharose but it contained covalently linked hemoglobin. With this support material proteins other than proteases

[1]Contribution No. 208 of the Department of Plant Science, University of Manitoba, Winnipeg 19, Manitoba, Canada, with financial assistance from the National Research Council of Canada.

passed directly through the column, whereas the enzymes were adsorbed and were subsequently eluted by changing the pH of the eluting buffer solution. The procedure was found very efficient for the purification of wheat proteases, and it might be applicable to other proteolytic enzyme systems.

MATERIALS

Sepharose 4B was obtained from Pharmacia, cyanogen bromide (MP 50-52°C) from Matheson Coleman & Bell Company, and hemoglobin (lyophilized, salt free) from Mann Research Laboratory Inc. Other chemicals used were of reagent grade and the distilled water was demineralized.

METHODS

Preparation of Enzyme Solutions

The starting enzyme solutions were obtained by extracting malted wheat flour with four volumes of 10% saturated ammonium sulfate solution (100 g flour; 400 ml of solution). This crude extract was immediately fractionated by precipitation with ammonium sulfate. The fraction that precipitated between 40-80% saturation of ammonium sulfate was collected and lyophilized. One gram of the lyophilized material was dissolved in 100 ml of the elution buffer containing 12.5 mg of L-cysteine mono-hydrochloride and 10 mg of di-sodium salt of ethylenediaminetetraacetic acid (EDTA). The solution was dialysed against two 2,000 ml volumes of the same buffer for 48 hours in the cold room (2-3°C). The insoluble residue which precipitated during dialysis was removed by centrifugation. Portions of the supernatant were then concentrated by ultra-filtration before application to the column.

Protein content of all solutions was determined by Lowry's method (1951) using gamma-globulin as standard. The protein in the eluents from chromatographic columns was measured as optical density at 280 mμ with spectrophotometer.

Protease Activity Determinations

Proteolytic activity was measured by the Ayre-Anderson method as

modified by McDonald and Chen (1964). In the assay used, 1.0 ml of the enzyme solution was added to 5 ml of 1% hemoglobin solution in 0.1N acetate buffer solution of pH 3.8. After two hours incubation at 37°C the reaction was stopped by adding 5 ml of 5% trichloroacetic acid (TCA). The reaction mixture was then centrifuged to remove the precipitate and 1 ml of the supernatant was withdrawn for the determination of the soluble peptides and amino acids by Lowry's procedure (1951).

Preparation of Substituted Sepharose

The procedure described by Cuatrecasas et al. (1968) was used to activate the Sepharose with cyanogen bromide. The Sepharose suspension (125 ml) was mixed with an equal volume of water, and 12.5 gm of cyanogen bromide, dissolved in 125 ml of water, was then added to the Sepharose. The pH was immediately adjusted to 11 by titration with 4N sodium hydroxide. After ten minutes, the activated Sepharose was washed with twenty volumes of cold 0.1N sodium bicarbonate solution on a Buchner funnel using mild suction. The washed activated Sepharose was suspended in 125 ml of 0.1N sodium bicarbonate solution of pH 9.0, and 50 ml of 4% hemoglobin solution was quickly added. This mixture was stirred gently at 2-3°C for 24 hours and washed extensively with water. Finally it was poured into the column and equilibrated with the buffer solution used to dissolve the crude, lyophilized enzyme preparation.

Disc Electrophoresis

Disc electrophoresis on 7.5% polyacrylamide gel was carried out at pH 4.0 as described by Davis (1964). The gels were stained overnight with 0.5% aniline blue black dissolved in 7% acetic acid solution. Destaining was carried out electrophoretically with 7% acetic acid as the electrolyte.

RESULTS AND DISCUSSION

When the crude enzyme solution was chromatographed on the unsubstituted Sepharose column (Fig. 1) the protein and the enzyme activity peaks were eluted with 0.05N acetate buffer of pH 5.5 at the same fraction number. No

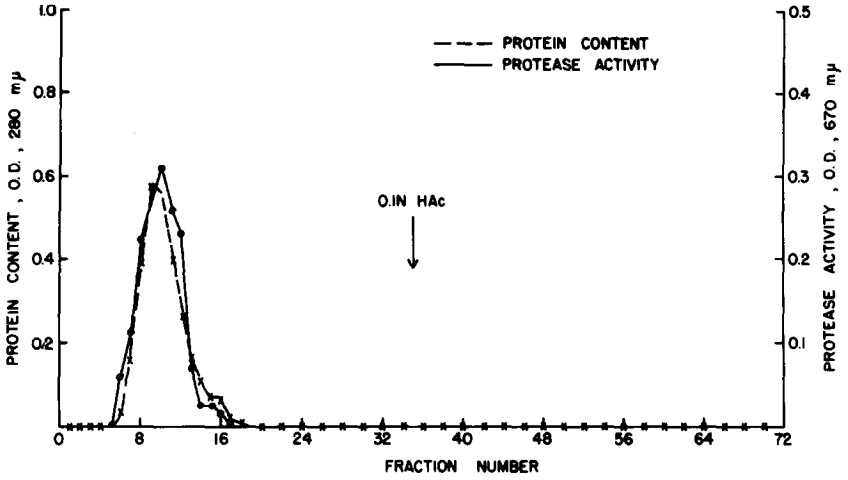

Fig. 1 Protein content and protease activity curves for fractions from crude extract separated on unsubstituted Sepharose using 0.05N acetate buffer of pH 5.5 as the starting eluent. Volume of extract used was 3 ml and elution flow rate was 50 ml per hr.

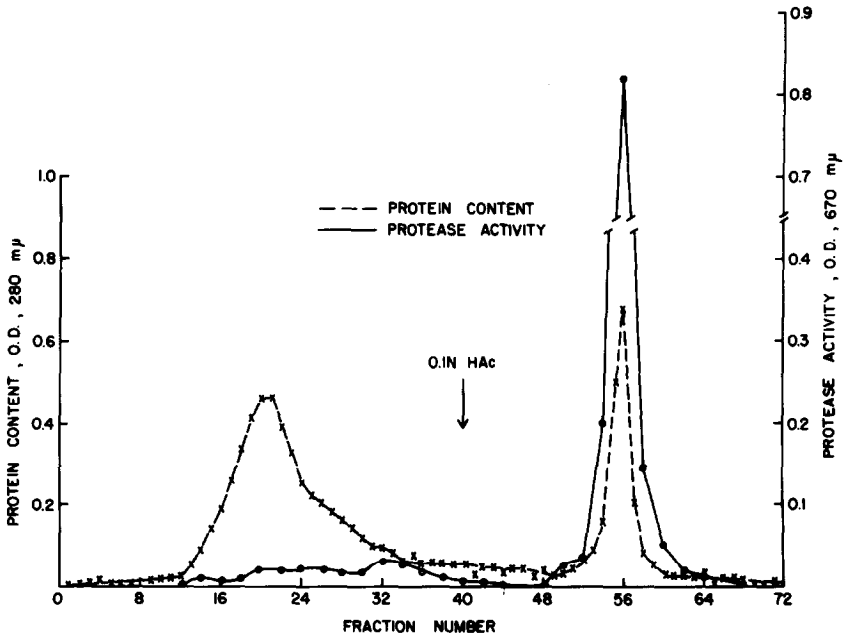

Fig. 2 Protein content and protease activity curves for fractions from crude extract separated on hemoglobin-substituted Sepharose using 0.05N acetate buffer of pH 5.5 as the starting eluent. Volume of extract used was 5 ml and elution flow rate was 50 ml per hr.

additional protein or enzyme activity was eluted when the eluting buffer was changed to 0.1N acetic acid of pH 3.1. The protein and enzyme activity recoveries were over 95% and were considered satisfactory.

Results obtained with the hemoglobin-Sepharose column are shown in Figure 2. About 55% of the protein was eluted with the starting buffer solution (pH 5.5) but essentially all of the proteolytic activity was retained on the column. The remaining protein (45%) and all of the enzyme activity was readily eluted with 0.1N acetic acid solution of pH 3.1. This fractionation produced a two-fold increase in specific activity. With partially purified extracts of proteolytic enzymes, this is considered a significant increase in activity.

The homogeneity of the proteolytically active protein fraction was examined by disc electrophoresis (Figure 3). The active peak still contains three to four distinguishable bands of slightly different mobilities. However,

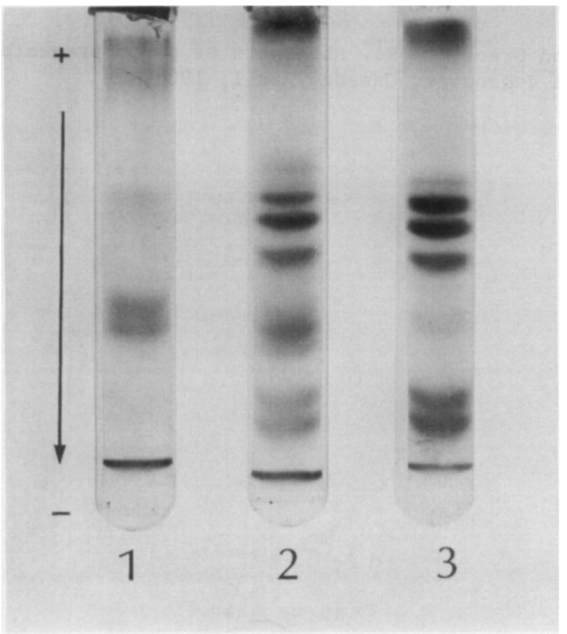

Fig. 3 Disc electrophoretic patterns for (1) the proteolytically active fraction, (2) the crude enzyme extract, and (3) the unadsorbed nonactive protein fraction obtained by fractionation on hemoglobin-Sepharose.

considerable purification was achieved as can be seen from a comparison of the pattern for the active peak (1) and the pattern for the crude enzyme solution used in the purification (2). The electrophoretic pattern for the protein peak eluted from hemoglobin-Sepharose with the starting buffer (3) has all the bands of the crude extract except the bands of the active peak.

ACKNOWLEDGEMENT

The authors wish to thank Miss P. Chiu for her technical assistance.

REFERENCES

Chua, G. K., and Bushuk, W. Abstracts of paper presented at 54th AACC meeting, Chicago, April, 1969

Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. Proc. Nat. Acad. Sci. (U.S.) 61, 636 (1968)

Davis, B. J., Ann. N. Y. Acad. Sci. 121, 404 (1964)

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. J. Biol. Chem. 193, 265 (1951)

McDonald, E. C. The Baker's Digest, 43, 26 (1969)

Wang, C. C., and Grant, D. R. Abstracts of paper presented at C.I.C. meeting at Vancouver, Canada, April, 1968.