Immobilized Metal Affinity Chromatography of Wheat α-Amylases[1]

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ABSTRACT

Cereal Chem. 65(5):413–416

An immobilized metal affinity chromatography technique for the purification of α-amylases from germinated wheat is described. Wheat α-amylases were retained on a column with Cu-iminodiacetic acid-epoxy-Sepharose 6B equilibrated with a 0.05*M* Tris-HCl buffer, pH 7.5, containing 0.15*M* NaCl and 10^{-4}*M* $CaCl_2$. A major portion of the contaminating proteins passed through in the equilibration buffer. Of the bound proteins, two groups of α-amylases were eluted from the column by the addition of increasing concentrations of glycine to the equilibration buffer. The group of α-amylases exhibiting low isoelectric points (pI) was eluted by the buffer containing 20 m*M* glycine, whereas the predominant group of high-pI α-amylases was eluted by the buffer containing 100 m*M* glycine. For the fraction eluted by the buffer containing 100 m*M* glycine, an increase in specific activity of approximately 20-fold with a recovery of 51.3% of the enzyme activity in the crude extract was obtained. The purity of the wheat α-amylase fractions was determined by isoelectric focusing followed by the β-limit dextrin plate technique used for the detection of α-amylase activity and protein staining.

Much attention has been paid to cereal α-amylases because of their important role in the process of degrading starch. Two main groups of α-amylases were shown to exist in germinated wheat (Olered and Jonsson 1970, Sargeant and Walker 1978, Kruger 1972, Marchylo et al 1980). The majority of total enzyme activity is contributed by a group of components with isoelectric point (pI) values of 6.0–6.5; the other group of α-amylase components is characterized with pI values of 4.5–4.8 (Sargeant and Walker 1978).

Published purification procedures for α-amylases (Kruger and Tkachuk 1969, MacGregor et al 1971, Tkachuk 1975, Silvanovich and Hill 1976, Sargeant and Walker 1978, Marchylo and Kruger 1983, Mundy et al 1983, Weselake and Hill 1983) are in most cases laborious, multistep procedures and usually result in low yields of enzyme. Thus the purpose of this study was to examine the feasibility of using immobilized metal affinity chromatography (IMAC), as a one-step purification method for the separation of wheat α-amylases.

IMAC is a separation technique based on a specific and reversible interaction between a metal immobilized on an insoluble matrix and a molecule of interest, e.g., a protein. The binding of a protein molecule to an immobilized metal ion occurs via the surface-exposed amino acid residues able to form coordination bonds. Desorption of proteins may be accomplished by stepwise or continuous change of pH or by a decrease of salt concentration. More selective desorption can be achieved by including in the eluent buffer solutes that effectively compete with the proteins for adsorption.

[1]Presented at the AACC 72nd Annual Meeting, Nashville, TN, November 1987. Contribution of ABI Biotechnology Inc., A-1150 Waverley Street, Winnipeg, Canada, R3T 0P4.

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This method has been developed by Porath and co-workers (Porath et al 1975, Porath and Olin 1983) and successfully used for the purification of different proteins including plasma proteins, interferons, nucleic acids, and several enzymes (reviewed by Lonnerdal and Keen 1982). For plant proteins, IMAC has been applied for the purification of *Dolichos biflorus* seed lectin (Borrebaeck et al 1981) and Jerusalem artichoke phenolase (Zawistowski et al 1987). IMAC of cereal proteins, however, has never been reported.

MATERIALS AND METHODS

Chemicals

Sepharose 6B, Pharmalytes, pH 4–6.5 and pH 3–10, and isoelectric focusing reference proteins for determination of isoelectric points in the pH range 5–10.5 were from Pharmacia, Canada Inc., Dorval, Quebec; iminodiacetic acid disodium salt, 1,4-butanediol diglycidyl ether, glycine, and sweet potato β-amylase were purchased from Sigma Chemical Company, St. Louis, MO. Acrylamide, *N,N'*-methylene-bisacrylamide, *N,N,N',N'*-tetramethylenediamine (TEMED), and ammonium persulfate were of electrophoresis grade and were obtained from Bio-Rad, Richmond, CA. Waxy maize starch was obtained from C. Biliaderis, Food Science Department, University of Manitoba. All other chemicals were of analytical reagent grade.

Purification Procedure

α-Amylase was extracted from wheat cultivar Neepawa germinated three days. The meal was slurried in 0.05*M* Tris-HCl buffer, pH 7.5, containing 0.15*M* NaCl and 10^{-4}*M* $CaCl_2$ (1:2.5, w/v) and mixed for 1 hr at 4°C using a magnetic stirrer.

All subsequent operations were performed at 4°C. The supernatant solution was adjusted with 1*M* NaOH to pH 7.5. Then min. Since the pH dropped slightly after extraction, the pH of the

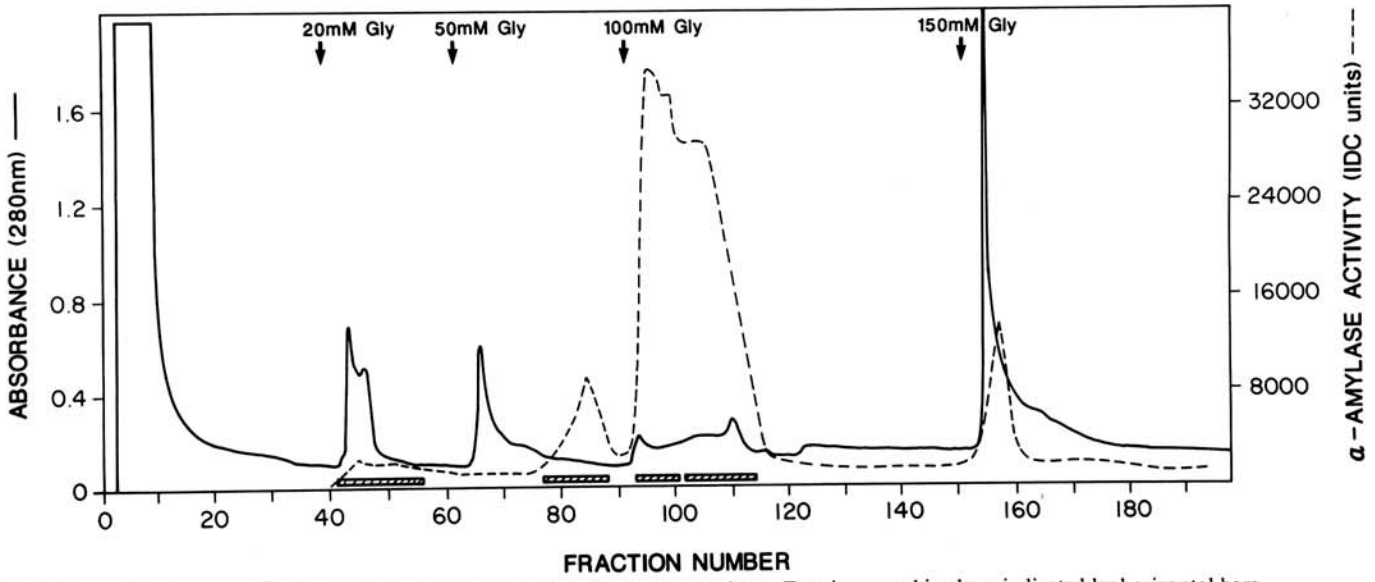

Fig. 1. Immobilized copper affinity chromatography of germinated wheat α-amylases. Fractions combined are indicated by horizontal bars.

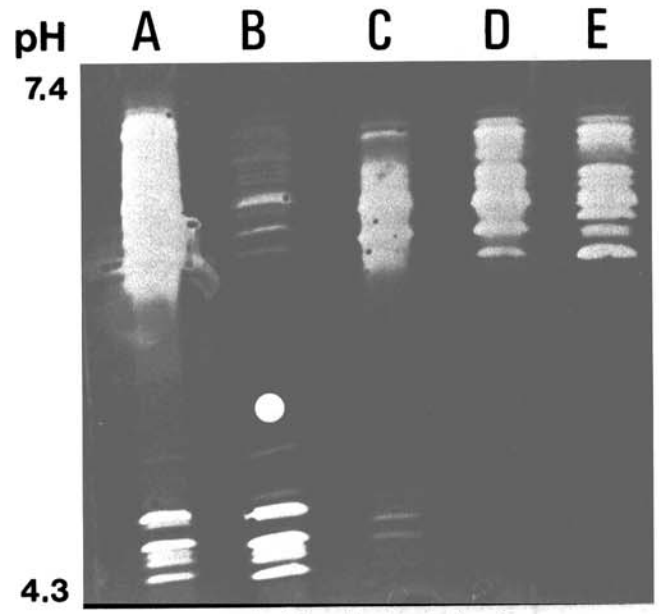

Fig. 2. Analytical polyacrylamide gel isoelectric focusing zymograms of α-amylase fractions; crude extract (**A**); Cu-column fractions (**B–E**): 20 m*M* glycine-eluted fractions 41–56 (**B**); 50 m*M* glycine-eluted fractions 77–88 (**C**); 100 m*M* glycine-eluted fractions 93–101 and 102–114, respectively (**D** and **E**). 100–150 iodine dextrin color units applied per lane.

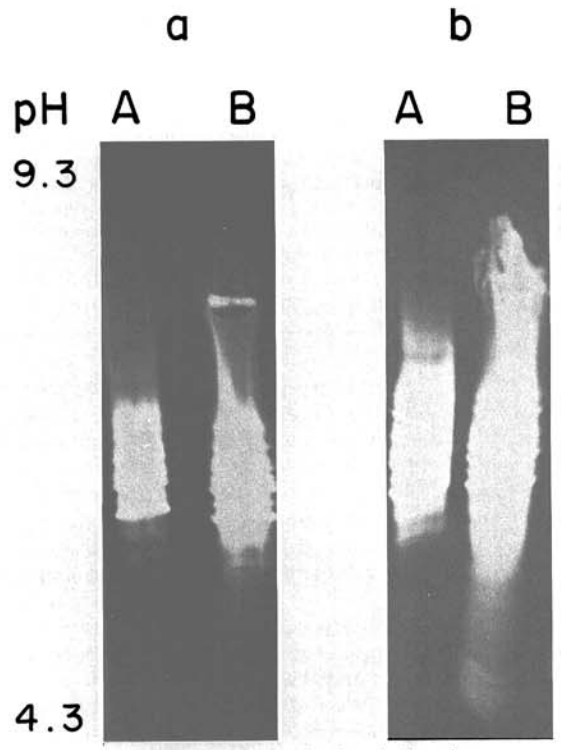

Fig. 3. Analytical polyacrylamide gel isoelectric focusing zymograms of α-amylase Cu-column fractions prepared with **a,** β-limit dextrin, or **b,** starch: 100 m*M* glycine eluted fractions 93–114 (**A**); 150 m*M* glycine-eluted fractions 153–162 (**B**). 100–150 iodine dextrin color units applied per lane.

supernatant solution was adjusted with 1*M* NaOH to pH 7.5. Then the solution was left for 1 hr at 4° C and centrifuged at 13,000 × *g* for 20 min to remove the formed precipitate. The clear supernatant was used for further purification by IMAC on copper-iminodiacetic acid-epoxy-Sepharose 6B (Cu-IDA-Sepharose 6B). Epoxy-Sepharose 6B was prepared using the method of Sundberg and Porath (1974), while the coupling of IDA to epoxy-activated Sepharose 6B was performed according to Porath et al (1975).

Prepared IDA-Sepharose 6B was packed into a two-column system consisting of a working column (1 × 14.5 cm) and a capture column (1 × 15.5 cm) coupled in sequence. Both columns were washed with six bed volumes of deionized water. The working column was then loaded with cupric sulfate solution containing 6 mg of $CuSO_4 \cdot 5H_2O$/ml until the blue color of copper ions was detectable in the eluate and then washed with six bed volumes of deionized water. The capture column was used to scavenge any copper ion that might leak from the working column during enzyme elution, thus preventing contamination of the enzyme preparation with copper ions. Both columns were separately equilibrated overnight with 0.05*M* Tris-HCl buffer, pH 7.5, containing 0.15*M* NaCl and 10^{-4}*M* $CaCl_2$ and then connected.

The supernatant from 26 g of germinated wheat, prepared as described above, was applied to the Cu-IDA-Sepharose/IDA-Sepharose 6B column system at a flow rate of 20 ml/hr; then the columns were eluted sequentially with equilibration buffer and equilibration buffers containing 20, 50, 100, and 150 m*M* glycine, respectively. Elution with each of these buffers was continued until absorbance at 280 nm was negligible. Fractions of 7.5 ml were collected. Columns were regenerated after each run by washing with solution containing 0.05*M* EDTA and 0.5*M* NaCl.

The column fractions containing α-amylase activity were analyzed by polyacrylamide gel isoelectric focusing followed by the detection of α-amylase activity using the β-limit dextrin plate technique (MacGregor et al 1974). Fractions containing α-amylase

TABLE I
Purification of Germinated Wheat α-Amylases by Immobilized Copper Affinity Chromatography

Fraction	Activity (IDC[a] units)	Protein (mg)	Specific Activity (IDC[a] units/mg)	Purification Factor (fold)	Yield (%)
Crude extract	997,304	858.00	1,162	1	100
Crude extract after pH adjustment	757,890	850.50	891	0.8	76.0
Immobilized copper affinity chromatography					
20 m*M* Gly, 41–56[b]	14,640	74.76	196	0.2	1.5
50 m*M* Gly, 77–86	64,542	50.22	1,285	1.1	6.5
100 m*M* Gly, 93–114	511,130	22.59	22,626	19.5	51.3
150 m*M* Gly, 153–162	54,168	ND[c]	ND	ND	5.4

[a] Iodine dextrin color.
[b] Column fractions combined. Gly = glycine.
[c] ND = Not determined.

activity were combined, concentrated using an Amicon cell (Amicon, Canada Ltd., Oakville, Ontario) equipped with 10,000 molecular weight cutoff membrane PM10, dialyzed against 0.02*M* sodium acetate buffer, pH 5.5, containing 10^{-3} *M* $CaCl_2$, and freeze-dried. Protein content was determined by the method of Lowry et al (1951) using bovine serum albumin as a standard, or by measuring absorbance at 280 nm.

Enzyme Assay

During purification, enzyme activity was assayed according to the method of Briggs (Briggs 1961) as modified by MacGregor et al (1971). A unit of activity (iodine dextrin color, IDC) was defined as the amount of enzyme required to change the absorbance of a β-limit dextrin solution from 0.6 to 0.4 in 100 min. The β-limit dextrin was prepared from waxy-maize starch as described by Kruger (1972).

Isoelectric Focusing

Isoelectric focusing (IEF) was conducted using 0.5-mm thick 7% polyacrylamide gels containing 12% sucrose and 6.3% of Pharmalytes, pH 3–10 and pH 4–6.5 mixed in a ratio of 15:4, respectively, using a maximum of 2,000 V with 6 W constant power at 10°C for approximately 2 hr. Gels were then stained for α-amylase activity using β-limit dextrin (MacGregor et al 1974) or starch (Tkachuk 1975) plates and for protein (Oakley et al 1980).

RESULTS AND DISCUSSION

The elution profile obtained on a Cu-IDA-Sepharose column is shown in Figure 1. The bulk of the proteins present in the crude extract did not bind to the column and passed through with the equilibration buffer. At least seven peaks were eluted by increasing the concentration of glycine in the equilibration buffer. Some of the α-amylase activity emerged with the buffers containing 20, 50, and 150 m*M* glycine, but most of the enzyme was eluted with the buffer containing 100 m*M* glycine.

Analysis of the eluent fractions by polyacrylamide gel IEF followed by detection of α-amylase activity using the β-limit dextrin plate technique (Fig. 2) showed that the application of the buffer containing 20 m*M* glycine removed mainly low-pI amylases from the column (Fig. 2, lane B), whereas the buffer containing 50 m*M* glycine eluted some high-pI α-amylases contaminated with low-pI α-amylases (Fig. 2, lane C). Although, at least two protein and two activity peaks were eluted with the buffer containing 100 m*M* glycine, all of the fractions contained essentially high-pI α-amylases and had identical composition, as detected by zymograms (Fig. 2, lanes D and E). Similarly, fractions eluted with the 150 m*M* glycine buffer contained only high-pI α-amylases (Fig. 3a, lane B).

Thus the separation of high- and low-pI α-amylases by IMAC was obtained by changing the concentration of glycine in the elution buffer. High-pI α-amylases, which constitute approximately 84% (Sargeant and Walker 1978) or 87–93% (Marchylo et al 1984) of the total germinated wheat α-amylases, were eluted mainly at the 100 m*M* glycine concentration. Earlier elution of low-pI α-amylases from the Cu-column indicate that their binding to immobilized copper was weaker than the binding of high-pI α-amylases. This result may be explained by the differences in the surface amino acids available for binding to the copper for the two groups of enzymes. Diverse Cu-binding capacities for high- and low-pI α-amylases could reflect known differences in their physicochemical and molecular properties characterized by others (Jacobsen et al 1970, MacGregor and Ballance 1980, Brown and Jacobsen 1982, Svensson et al 1985).

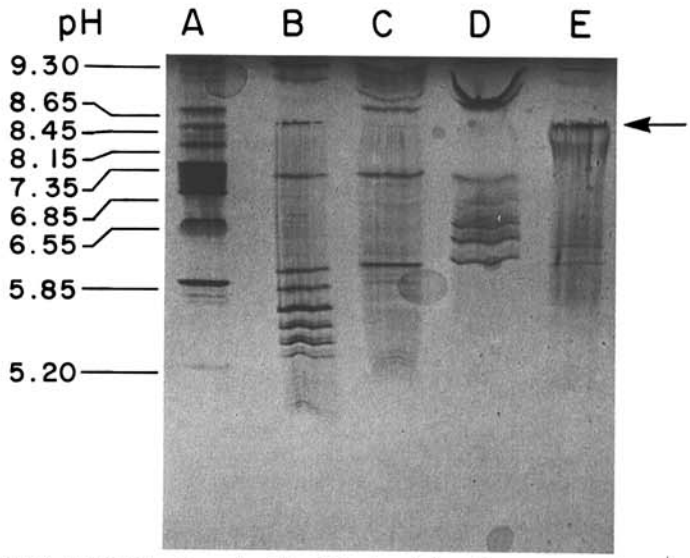

Fig. 4. Analytical polyacrylamide gel isoelectric focusing patterns stained for protein; pI marker proteins (**A**); α-amylase crude extract (**B**); α-amylase Cu-column fractions (**C**–**E**): 20 m*M* glycine-eluted fractions 41–56 (**C**); 100 m*M* glycine-eluted fractions 93–114 (**D**); 150 m*M* glycine-eluted fractions 53–162 (**E**). Approximately 40 μg of protein applied per lane. Arrow indicates position of sample application.

Polyacrylamide gel IEF patterns of α-amylase fractions stained for protein are shown in Figure 4. The major protein bands in the pH region 6.0–7.3 obtained for the combined column fractions eluted with 100 m*M* glycine (Fig. 4, lane D) correspond to the high-pI α-amylase bands observed in the zymogram (Fig. 2, lanes D and E). A strong contaminating protein band is present in the approximate region of pH 9.0 of this pattern. The minor α-amylase fractions eluted with 20 m*M* (Fig. 4, lane C) and 150 m*M* (Fig. 4, lane E) glycine exhibit a broad spectrum of pI values. The low degree of purity obtained for these fractions is reflected also by their low specific activities (Table I).

A major difficulty in purifying α-amylase from cereal grains is removing β-amylase activity from the α-amylase preparations. To follow the fate of β-amylase during IMAC, differentially eluted combined Cu-column fractions were analyzed by IEF-zymograms using starch as a substrate. The presence of additional bands in the starch zymogram of 150 m*M* glycine-eluted Cu-column fractions (Fig. 3b, lane B) and lack of these bands in the zymogram for the

same fractions obtained with β-limit dextrin plate (Fig. 3a, lane B) suggest the presence of β-amylase in the fractions eluted with 150 m*M* glycine.

The Cu-column fractions eluted with 100 m*M* glycine containing high-pI α-amylases gave basically the same zymograms with starch (Fig. 3b, lane A) and β-limit dextrin (Fig. 3a, lane A) obtained after IEF. Thus the 100 m*M* glycine-eluted high-pI α-amylases fraction was free of contamination with β-amylases.

Results of the purification of the germinated wheat α-amylases are shown in Table I. The specific activity of α-amylases in the crude extract was 1,162 IDC units per milligram of protein. During the pH adjustment, approximately one-fourth of the α-amylase activity was lost. Thus only about three-quarters of the α-amylase activity present in the crude extract was applied to the Cu-column. For the major activity peak including fractions 93–114 eluted with 100 m*M* glycine, an approximately 20-fold increase in specific activity with a recovery of 51.3% of the enzyme activity in the crude extract or 67.4% of that in the pH-adjusted crude extract was obtained. The purification factor for the minor α-amylase fractions (eluted with 20, 50, and 150 m*M* glycine) was poor due to the concomitant elution of significant amounts of other proteins. Approximately 85% of the total α-amylase activity applied to the column was recovered in all column fractions. About 80% of the recovered activity was present in the 100 m*M* glycine-eluted fraction. As demonstrated by the activity recovery data (Table I) and by the zymograms (Fig. 2), immobilized copper affinity chromatography resulted in an active, nondenatured enzyme. Although IEF patterns of the major activity fraction (high-pI α-amylases) exhibited numerous contaminating proteins, a substantial degree of purification was achieved by IMAC for this fraction. Moreover, IMAC-purified high-pI α-amylases, constituting approximately 67% of the total α-amylase activity applied to the metal affinity column, were free of low-pI isoenzymes as well as β-amylases.

In the experiment described here, the total amount of protein applied to the column was approximately 77 mg/ml of sedimented gel. Studies with increasing amounts of α-amylase extract applied to the Cu-IDA-Sepharose 6B column indicated that the gel capacity is approximately 180 mg protein or 2.7×10^5 IDC units/ml of gel (an average calculated from six different experiments). The high capacity of the gel makes IMAC a suitable method for a large-scale purification of germinated wheat α-amylases. The method enables the separation of two groups of α-amylases in a single-step chromatographic procedure with a high recovery of the native high-pI enzymes. Moreover, IDA-Sepharose gel can be easily regenerated, and its high stability allows for repetitive use of the columns. Thus IMAC is a simple, reproducible, and inexpensive method.

Because IMAC utilizes other protein properties than those commonly used, such as adsorption, molecular size, surface charge, etc., this method may become the method of choice where conventional separation techniques have proven inadequate with regard to recovered activity or yield.

ACKNOWLEDGMENT

We thank C. Biliaderis, Food Science Department, University of Manitoba, for providing the waxy maize starch used in this study.

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[Received February 1, 1988. Accepted May 9, 1988.]

ERRATUM

Cereal Chemistry, Vol. 65:No. 5
(September–October 1988)

On page 413, in Materials and Methods, the second paragraph under the subheading "Purification Procedure" should begin:

All subsequent operations were performed at 4°C. The supernatant was collected by centrifugation at 13,000 × *g* for 20 min. Since the pH dropped slightly after extraction, the pH of the supernatant solution was adjusted with 1*M* NaOH to pH 7.5.