COMPETITIVE AFFINITY CHROMATOGRAPHY OF WHEAT α-AMYLASE

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1. Introduction

A simple and rapid technique, termed 'competitive affinity chromatography', is presented for isolation of wheat α-amylase. In this technique a crude enzyme extract is applied to an immobilized substrate (glycogen-Sepharose) and the α-amylase then eluted with a soluble substrate (glycogen). Wheat α-amylase can be obtained with a protein purity of approximately 90% and with an activity recovery of 84%.

2. Experimental

2.1. *Glycogen*

Glycogen (Shellfish, Type II, Sigma Chemical Co.) was purified by passing a 0.5% solution through a DEAE-cellulose (hydroxide form) bed on a Buchner funnel and freeze-drying the colorless filtrate.

2.2. *Glycogen-Sepharose*

Glycogen was coupled to Sepharose in a similar manner to the method used by Kagedal and Akerstrom for preparing soluble protein-dextran derivatives [1]. To a stirred solution of 0.5 g glycogen in 800 ml water at 21–22°C, 0.5 g BrCN was added, and the Ph was adjusted and maintained at pH 10.7 for 2 hr with an automatic titrator (radiometer Titrator Type TT1B-Titrigraph Type SBR2c). Next, 1 g of pre-swollen CH-Sepharose-4B (Pharmacia Fine Chemicals) in 50 ml of 0.5 M NaCl was added to the activated glycogen, and the resulting mixture was maintained at pH 10.7 for and additional 2 hr. Then, 100 ml of 1 M ethanolamine and 5 g of $NaHCO_3$ were added and the mixture was stirred gently overnight with an overhead stirrer. The resulting gel was poured into a 2 cm diameter column and washed with approx. 10 bed vol of 0.5 M sodium acetate pH 5.5 and finally equilibrated with 0.005 M calcium acetate pH 5.5.

2.3. *Preparation of extract*

A 200 g ground sample of 5-day germinated red spring wheat (cultivar Manitou) was dispersed with 600 ml 0.005 M calcium acetate pH 5.5 in a Waring Blendor. The dispersion was centrifuged, and the supernatant was heated at 70°C for 10 min to destroy the β-amylase. The heat-treated mixture was centrifuged and the supernatant used directly for the isolation of α-amylase.

2.4. *α-Amylase assay*

The method of Bernfeld using, 3,5-dinitrosalicylic acid [2] as modified by Tkachuk and Kruger [3] was used to measure the α-amylase activity.

3. Results and discussion

Competitive affinity chromatography of wheat α-amylase is shown in fig.1. Proteins, and enzymes other than α-amylase are not adsorbed and pass through the affinity gel column. The α-amylase is eluted from the gel with 0.4% glycogen, and accordingly this double affinity chromatography procedure has been termed 'competitive affinity chromatography'. The adsorbed α-amylase could not be eluted from the gel with 0.005 to 1 M acetate or Tris buffers ranging from pH 5.0 to 9.2, or with 0.4% glycogen containing Ca^{++}. Polyacrylamide gel electrophoresis of the α-amylase purified by competitive affinity chromatography revealed (fig.2A) three major bands and one very faint protein band. Previous studies have shown that

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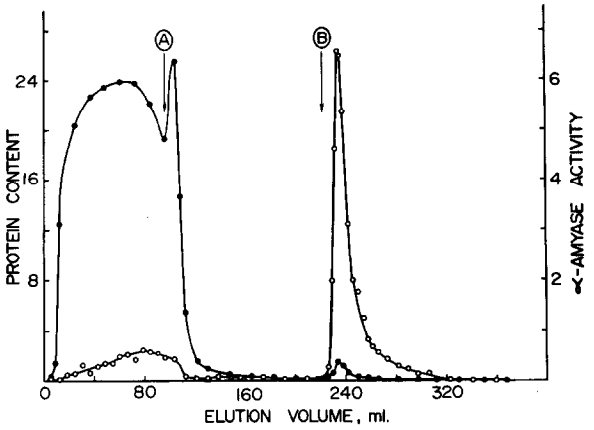

Fig.1. Competitive affinity chromatography of wheat α-amylase on glycogen-AH Sepharose 4B. A crude extract (96 ml) containing 600 mg protein and 6.45 mg α-amylase was applied into a 20 × 30 mm column of gel equilibrated with 0.005 M sodium acetate–0.005 M calcium acetate, pH 5.5. The column was then washed (at point A) with approx. 40 ml 1 M Tris–0.005 M calcium acetate, pH 8.6, followed by a minimum of 100 ml 1 M sodium acetate–0.005 calcium acetate, pH 5.5. The α-amylase was removed from the column (at point B) with 0.4% glycogen. Flow rates were approximately 100 ml/hr (gravity flow) and 5 ml fractions were collected. The chromatography was carried out in a cold room at 3°C. Protein was monitored by absorption at 280 nm (1 cm cells) (–●–●–). Alpha-amylase activity was monitored by measuring reduced dinitrosulfosalicylic acid at 497 nm (–o–o–). A 84% recovery of α-amylase was obtained.

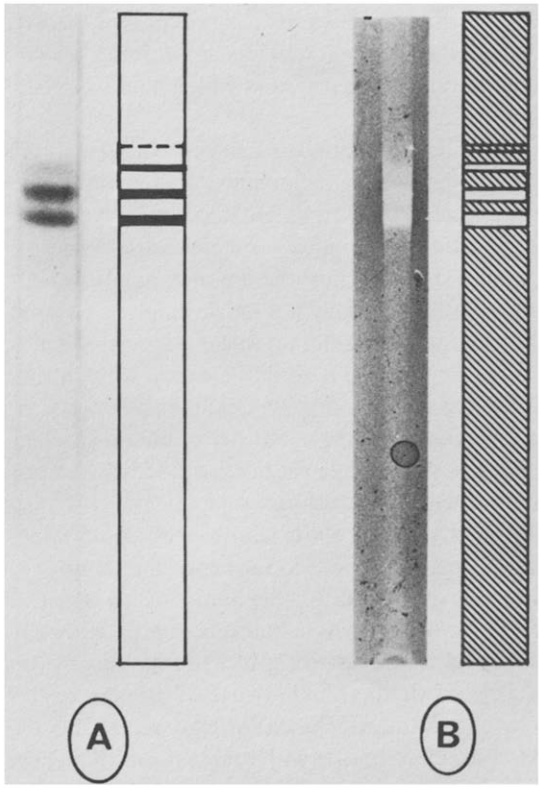

Fig.2. Polyacrylamide gel electrophoresis of approx. 20 μg wheat α-amylase purified by competive affinity chromatography. A. Protien bands obtained on electrophoresis. Gel stained with Coomassie Blue. B. Enzyme zymogram obtained by sandwiching the electrophoresis gel shown in A (before staining for protein) with another flat polyacrylamide gel containing 0.1% starch, and subsequently staining the flat gel with I_2/KI solution. The colorless areas show locations where starch has been hydrolyzed by α-amylase.

four α-amylase isoenzymes are present in wheat [3]. Sandwiching the polyacrylamide electrophoresis gels with flat polyacrylamide gels containing soluble starch, showed that the protein bands possessed α-amylase activity (fig.2B).

The purified α-amylase had a specific activity of approximately 1300 units/mg protein* compared to 15 units/mg protein in the extract applied to the column. Thus, a 93-fold purification can be achieved in this single purification step. Lower specific activities are obtained if the affinity column is not washed thoroughly before the elution of α-amylase, and also if Ca^{++}salts are not added to the eluted α-amylase. In the absence of Ca^{++}, purified α-amylase denatures quite quickly. The major impurity in the α-amylase effluent from the affinity column is glycogen. Some of the glycogen can be removed (after digestion by the α-amylase) by dialysis or ultra-filtration; however, all of the glycogen can be removed by DEAE-chromatography, a procedure which also serves to separate the α-amylase isoenzymes [3].

Three successive experiments on the same affinity column gave an α-amylase recovery of 85, 84 and 82% of the amount applied to the column, indicating that the affinity gel can be used repeatedly and with good α-amylase recoveries. Repeated use of the gel

* One unit is equivalent to the reducing power of 1 μmol maltose per min. One mg of pure wheat α-amylase has an activity equivalent to approx. 14 μmol maltose min^{-1} at 25°C [3].

is only possible, however, if freshly prepared extracts are used, as older wheat extracts can develop brown colored polyphenolic materials which bind irreversibly onto the gel.

Relatively pure α-amylase can be isolated in approx. 6 hr using the affinity technique. Pure individual α-amylase isoenzymes can be obtained in 2 days if one uses the affinity technique combined with ion exchange procedures. Thus, the use of competitive affinity chromatography is a much simpler and more rapid technique for isolating wheat α-amylase than the more lengthy and laborious classical techniques [4]. Scaling up the affinity technique should present no problems, thus large quantities of pure α-amylase should now be available for much needed characterization studies of this enzyme.

The best yields of α-amylase were obtained when glycogen was complexed to the six-carbon atom 'spacer arm' present in AH-Sepharose 4B. It is not known whether α-amylase binds more efficiently to glycogen when the latter is attached to a 'spacer arm' [5], or whether the presence of the 'spacer arm' merely allows the attachment of glycogen to the Sepharose. Experiments with preparations of glycogen attached to agarose or cellulose showed that these materials did not bind significant amounts of α-amylase. Alpha-amylase was bound by a preparation of glycogen attached to ethylamine-cellulose. However, other proteins were bound also, indicating perhaps that not all of the ethylamine groups had reacted with glycogen or ethanolamine during the synthesis of the glycogen-ethylaminecellulose.

The technique of competitive affinity chromatography using immobilized glycogen should apply to α-amylases in general, since α-amylases from widely differing sources are known to complex with glycogen [6]. The principle in competitive affinity chromatography of using a soluble substrate (or inhibitor) to remove a bound enzyme should apply also to the isolation of other enzyme types, as the main difficulty of the simple affinity chromatography procedure is often the removal of a tightly bound enzyme from the affinity column.

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References

[1] Kagedal, L. and Akerstrom, S. (1971) Acta Chemica Scand 25, 1855–1859.
[2] Bernfeld, O. (1955) in: Methods in Enzymology (Colowick, S. O. and Kaplan, N. O., ed.) Vol. 1, 149, Academic Press, New York.
[3] Tkachuk, R. and Kruger, J. E. (1974) Cereal Chem. 51, 508–529.
[4] Kruger, J. E. and Tkachuk, R. (1969) Cereal Chem. 46, 219–226.
[5] Cuatrecasas, P. (1972) Advances in Enzymol. (Meister, A. ed.) 36, pp 29–89, Interscience.
[6] Loyter, A. and Schramm, M. (1962) Biochim. Biophys. Acta 65, 200–206.