View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

AFFINITY CHROMATOGRAPHY OF *SORGHUM* ACID PROTEASE

T.K. VIRUPAKSHA* and K. WALLENFELS
Lehrstuhl Biochemie, Universität Freiburg, 78 Freiburg i.Br. Albert Str. 21, GDR

Received 28 January 1974

1. Introduction

Sorghum acid protease is an endopeptidase which hydrolyzes peptide linkages involving the α-carboxyl group of either glutamic acid or aspartic acid [1]. Purification of this enzyme to near homogeneity by the conventional methods of enzyme fractions has been described [2]. In view of the potential usefulness of this protease in protein sequencing studies, it is desirable to develop a simple and rapid procedure for the purification of the enzyme.

Purification of enzymes and other proteins by affinity chromatography has become the method of choice in recent years [3]. It is based on the unique biological property of the proteins to interact specifically with ligands immobilized on an insoluble solid matrix. It has been observed that *N*-acetyl-D-glutamyl-D-glutamic acid is a competitive inhibitor of the *Sorghum* acid protease and the inhibition is pH-dependent [4]. This paper describes the purification of the *Sorghum* protease on affinity columns using D-Glu–D-Glu–Sepharose as an affinity ligand.

2. Methods

The procedure described by Cuatrecasas et al. [3] was followed for the activation of agarose and the coupling of the ligand. To 8.0 ml of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala) 8.0 ml of H_2O was added and stirred with 1.5 g of CNBr. The pH was raised to 11.0 by adding 4 N NaOH and maintained at this pH for 10 min. The activated agarose was coupled with 0.18 mmoles of D-Glu–D-Glu (cyclo Chemical Corp., Los Angeles) in 0.1 M sodium borate buffer, pH 9.0. Reaction was carried out at 4°C for 16 hr. The gel was filtered under suction and washed on the filter H_2O and then with 300 ml of 0.01 M sodium citrate buffer, 0.1 mm in EDTA, pH 3.7. An aliquot of the gel was hydrolyzed with 6 N HCL and the amount of ligand bound to the gel was determined by automated amino acid analysis.

Affinity chromatography was performed at 0°C on 10 mm × 70 mm gel colums. The column was equilibrated with 0.01 M citrate–EDTA buffer, pH 3.7. After protein was adsorbed to the column, nonadsorbed protein was removed by washing with 32.0 ml of the equilibration buffer. Elution was continued thereafter with 0.1 M citrate buffer, 0.1 mM in EDTA, pH 5.8.

Protein in effluent fractions was located by measuring the absorption at 280 nm. Protein estimations were done by the method of Lowry et al. [5].

Proteolytic activity was assayed by the standard procedure described earlier [2] using bovine serum albumin as the substrate.

A partially purified preparation of the *Sorghum* acid protease which was used as the starting material for the affinity chromatography, was obtained as described earlier [2]. However, batchwise adsoprtion to and desorption from DEAE-cellulose was employed here rather than DEAE-cellulose column procedure (Step IV, ref. [2].

Isoelectric focusing of protein samples in polyacrylamide gel (pH 5–8) was carried out as described by Wrigley [6] and Coomassie Brilliant Blue R250 was used as the protein stain.

* Present address: Department of Biochemistry, University of Agricultural Sciences, Hebbal, Bangalore 560024, India.

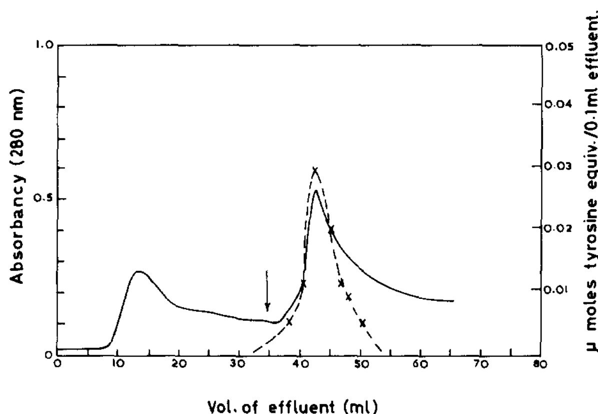

Fig. 1. Affinity chromatography of *Sorghum* acid protease on D-Glu–D-Glu–Sepharose. A sample (4.0 mg in 1.2 ml buffer) of the partially purified enzyme was applied to a 10 mm × 70 mm column of the gel equilibrated with 0.01 M citrate buffer, 0.1 mM in EDTA, pH 3.7, and the column was developed with the same buffer. At the point indicated by the arrow, change over to 0.1 M citrate–EDTA buffer, pH 5.8 was effected. Fractions (2.0 ml) were collected at a flow rate of 16 ml/hr. Absorption at 280 nm (- - -) and enzymic activity (x–x–x).

3. Results and discussion

Fig. 1 shows the affinity chromatography pattern of the *Sorghum* acid protease on D-Glu–D-Glu–Sepharose column. The amount of ligand bound per ml of packed gel was found to be 2 μmoles, Enzymically inactive protein is not adsorbed and is washed off the column with the pH 3.7 buffer: The enzyme which is adsorbed to the gel is detached from the adsorbent by the pH 5.8 buffer and is eluted from the column as a single protein and activity peak. The enzyme thus purified had a sp. act. of 800 units/mg protein compared to a sp. act. of 400 units/mg protein of the partially purified enzyme applied to the column. Recovery of enzymic activity was 96% of that applied to the column. The gel could be used repeatedly for affinity chromatography without loss of resolution or recovery.

Isoelectric focusing in polyacrylamide gel (pH range 5–8) of the *Sorghum* acid protease purified by affinity chromatography revealed a single protein band (fig. 2).

The successful purification of the *Sorghum* by affinity chromatography on D-Glu–D-Glu–Sepharose demonstrates that the enzyme is bound at pH 3.8 to the affinity ligand immobilized on agarose. This binding is perhaps related to the function of the ligand (D-Glu–D-Glu) as a pseudosubstrate. It is likely that ionic interactions of a group (or groups) at or near the active site of the enzyme, are responsible for the binding of the enzyme to the ligand. This interaction is weakened or abolished at higher pH (around pH 6.0). Therefore, D-Glu–D-Glu may be considered to be a biologically specific ligand of the *Sorghum* protease.

Fig 2. Isoelectric focusing in polyacrylamide gel of the *Sorghum* acid protease. A sample (100 μg) of the enzyme purified by affinity chromatography was added to the gel and run for 3 hr at a current strength of 2 mA per gel tube. Migration is from top pH 5 to bottom (pH 8).

It is a common observation in affinity chromatography that interposing an extension 'arm' of a hydrocarbon chain between the matrix and the ligand markedly increases the binding of the enzyme to its competitive inhibitor [4]. Steric interference that results

when the ligand is attached too closely to the supporting gel is believed to be responsible for this weak interaction [7]. It has been shown in the present study that attachment of D-Glu–D-Glu to agarose, without interposing an 'extension' between the gel and the ligand, results in a rather tight binding of the enzyme to the ligand. Presumably, the dipeptide ligand has a sufficient extension arm length to overcome the steric interference of the matrix on the ligand–enzyme interaction.

Affinity chromatography of the *Sorghum* acid protease on D-Glu–D-Glu–Sepharose gel provides a simple and rapid method of purification of the enzyme for protein sequencing studies and other investigations.

Acknowledgement

One of the authors (T.K.V.) is indebted to the Alexander von Humboldt Foundation of the Federal Republic of Germany for the award of a fellowship (1972–1973).

References

[1] Garg, G.K. and Virupaksha, T.K. (1970) Eur. J. Biochem. 17, 13.
[2] Garg, G.K. and Virupaksha, T.K. (1970) Eur. J. Biochem. 17, 4.
[3] Cuatrecasas, P. and Anfinsen, C.B. (1971) in: Methods in Enzymology (Jakoby, W.B., ed.) Vol. 22, p. 345, Academic Press, New York.
[4] Virupaksha, T.K. Unpublished results.
[5] Lowry, H.C., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.
[6] Wrigley, C. (1968) Sci. Tools, 15, 17.
[7] Cuatrecasas, P. (1970), J. Biol. Chem. 245, 3059.