BIOSPECIFIC AFFINITY CHROMATOGRAPHY OF PULLULANASE

Bent Stig ENEVOLDSEN, Lars REIMANN* and Niels Laurits HANSEN
Carlsberg Research Center, Department of Brewing Chemistry, Gamle Carlsberg Vej 10, DK-2500, Valby, Copenhagen, Denmark

Received 23 March 1977

1. Introduction

Biospecific affinity chromatography is a method of purification based on biospecific adsorption and subsequent desorption. The method relies upon a suitable biospecific ligand, a procedure for immobilizing the ligand in question, and a means of desorption, preferably using a counter-ligand.

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) is an enzyme capable of hydrolysing the 1,6-α-glucosidic linkages in pullulan to give maltotriose as the final product. The enzyme is produced by several bacteria and is also present in higher plants. Its ability to hydrolyse 1,6-α-linkages in branched dextrins is of importance in the structural analysis of amylopectin and glycogen and of potential interest in the industrial conversion of starch. The enzyme from *Aerobacter aerogenes* has been purified and crystallized [1–4] using conventional methods. In the present context it is of interest that Marshall [5] has reported that pullulanase is strongly inhibited by Schardinger α-dextrin and Schardinger β-dextrin with K_i 2.91×10^{-6} M and K_i 2.15×10^{-7} M, respectively.

This communication describes the purification of pullulanase from *Aerobacter aerogenes* by biospecific affinity chromatography. Schardinger α-dextrin was immobilized using a procedure somewhat similar to Kristiansen et al. [6]. The method which we have used involves immobilization of the soluble CNBr-activated Schardinger α-dextrin to the free amino groups of an insoluble support (AH-Sepharose 4B). After adsorption onto the affinity column pullulanase was released using Schardinger β-dextrin as the biospecific eluant. Finally, column chromatography on Sephadex G-15 was used for the separation of pullulanase and the counter-ligand.

Crossed immunoelectrophoresis [7] using antibodies towards crude pullulanase was used to follow the various steps of purification, since this technique permits observation of the (antigenic) impurities as well as pullulanase, even in the presence of the inhibitory ligands.

2. Materials and methods

Pullulanase was obtained as a crude powder (95 U/g), resulting from a salt precipiation of the extracellular medium of an *Aerobacter aerogenes* culture [8]. Antibodies against this preparation of crude pullulanase as well as towards purified pullulanase were prepared by Dakopatts A/S, Denmark, by immunization of rabbits. AH-Sepharose 4B and Sephadex G-15 were obtained from Pharmacia. Schardinger α-dextrin, free of Schardinger β-dextrin, was obtained from Sigma, Schardinger β-dextrin was from Pierce and CNBr from Aldrich. All other chemicals were readily available and of analytical grade.

The equipment for immunoelectrophoresis was from Dansk Laboratorieudstyr A/S. Agarose used for immunoelectrophoresis was from Litex, Denmark. Crossed immunoelectrophoresis was performed as by Weeke [7] using 1.5 mm gel thickness. Precipitates were stained with Coomassie brilliant blue R-250. The pullulanase precipitate was identified by two methods: by its ability to hydrolyse pullulan which had been

*Present Address: Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38101, USA

incorporated into the antibody-containing gel since the antigen-antibody precipitate (before staining) retains the enzymic activity, and by using antibodies towards purified pullulanase.

The specific pullulanase activity was determined on the basis of its activity [9] and the amount of protein [10]. The Schardinger dextrins were determined by quantitative gel-filtration chromatography [11] and by the phenol–sulphuric acid method [12].

2.1. *Immobilization of Schardinger α-dextrin*

The hydroxyl groups of the cyclodextrin were activated by means of CNBr in an aqueous solution at room temperature. The reaction mixture was kept at pH 10.5 by adding 1 N NaOH. When the consumption of NaOH ceased, the activation process was considered to be finished. The activated Schardinger α-dextrin was then added dropwise to a suspension of pre-washed AH-Sepharose 4B in 50 mM sodium borate buffer, pH 8.7. The coupling reaction was allowed to proceed overnight at 4°C. The gel with coupled Schardinger α-dextrin was then washed extensively on a sintered glass funnel with 75 mM sodium borate buffer, pH 8.7, containing 1 M NaCl and subsequently with 75 mM citrate-phosphate buffer, pH 5.0, containing 1 M NaCl.

The immobilization was carried out using 80 μmol Schardinger α-dextrin and approx. 160 μmol CNBr/g (dry) AH-Sepharose 4B. The difference between the amount of Schardinger α-dextrin used for activation and that found in the combined washings indicated that approx. 30 μmol Schardinger α-dextrin was immobilized/g AH-Sepharose 4B. According to the manufacturer, one gram AH-Sepharose 4B corresponds to 4 ml gel and contains 24–40 μmol reactive amino groups.

2.2. *Biospecific adsorption of pullulanase onto affinity column with Schardinger α-dextrin*

The prepared gel with its immobilized Schardinger α-dextrin was equilibrated with 30 mM citrate–phosphate buffer, pH 5.0 and packed into a column (Pharmacia K 16/40, bed volume 20 ml). All subsequent operations were performed at room temperature.

The crude pullulanase powder was suspended in 30 mM citrate–phosphate buffer, pH 5.0 containing 0.05% NaN_3 to prevent bacterial growth. The suspension was centrifuged and filtered. The solution contained 17.3 U/ml (0.43 U/mg protein). Its conductivity was 29 mMHO corresponding to 0.3 M NaCl. Of this solution 440 ml, corresponding to 7500 units of pullulanase, was fed to the affinity column. The affinity column was then washed with 30 mM citrate–phosphate buffer, pH 5.0, containing 0.4 M NaCl, in order to remove non-biospecifically adsorbed material. The washing (550 ml) was maintained until protein no longer was released from the column. Up to this point pullulanase activity could not be detected in the eluate.

A control experiment showed that under the above conditions pullulanase was not retained on underivatized AH-Sepharose 4B. However, if the ionic strength of the eluent was low, i.e., less than 0.3 M NaCl, pullulanase was also retained on underivatized AH-Sepharose 4B, probably due to ionic interactions.

2.3. *Biospecific desorption of pullulanase using Schardinger β-dextrin*

Desorption of pullulanase from the affinity column was performed using 1% Schardinger β-dextrin as biospecific competitive ligand in 30 mM citrate–phosphate buffer, pH 5.0, containing 0.4 M NaCl. Monitoring of the ultraviolet-absorbance revealed that protein was released from the affinity column and emerged as a narrow peak in a volume of about 35 ml.

2.4. *Removal of salts and Schardinger β-dextrin from purified pullulanase by means of Sephadex G-15 column chromatography*

The eluate, containing pullulanase as well as salts and the inhibitory Schardinger β-dextrin, was after passing the ultraviolet-monitor run directly to a Sephadex G-15 column (Pharmacia K 26/100, bed volume 450 ml). Sephadex G-15 was chosen specially for this purpose, since it had been reported [13] that Schardinger β-dextrin behaves anomalously on Sephadex G-15 in that it is retarded well beyond its expected elution volume. When the eluate from the affinity column had entered the Sephadex G-15 column, the Sephadex G-15 column was eluated with 10 mM phosphate buffer, pH 6.8. Pullulanase was excluded by the gel and well separated from the Schardinger β-dextrin. The solution of pullulanase contained 37 U/ml (30 U/mg protein).

3. Results

Crossed immunoelectrophoresis was used to characterize the crude pullulanase preparation. Figure 1 shows the many antigenic components present in the starting material. Among these precipitates the peak indicated by P presents the pullulanase protein. This was revealed by incorporating pullulan into the antibody-containing gel before the second-dimension electrophoresis. Among the precipitates formed, only peak P was able to hydrolyse pullulan. Furthermore, crossed immunoelectrophoresis of crude pullulanase towards antibodies against purified pullulanase revealed only one major peak, corresponding to peak P.

The adsorption of pullulanase onto the affinity column was followed by crossed immunoelectrophoresis of samples withdrawn from the eluate during the adsorption step and subsequent washing. Figure 2 shows the components present in the eluate after 400 ml crude pullulanase has passed through the affinity column (bed volume 20 ml). At this point all the components present in the crude pullulanase preparation (except peak P in fig.1) leave the affinity column in the same concentration at which they enter

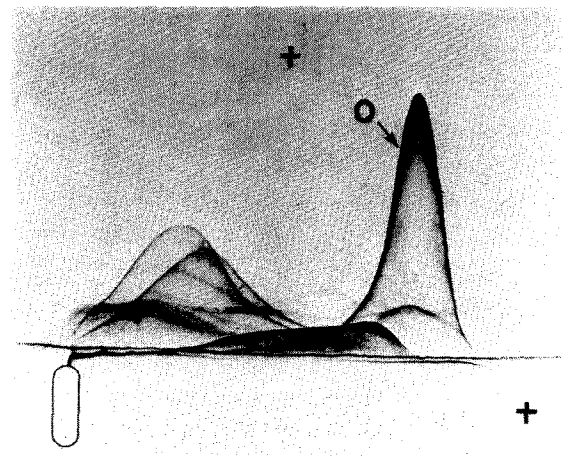

Fig.2. Crossed immunoelectrophoresis of eluate from affinity column. Antigen: 30 μl eluate from affinity column. At this stage of the adsorption process 400 ml of crude pullulanase had passed through the affinity column (bed vol: 20 ml). First-dimension electrophoresis: 100 min, 6.8 V/cm, 8°C. Antibodies: 18.6 μl/cm² gel area, against crude pullulanase. Otherwise, conditions as in fig.1. O: Component which was partially retained by the affinity column in the early stages of the adsorption process (see also text).

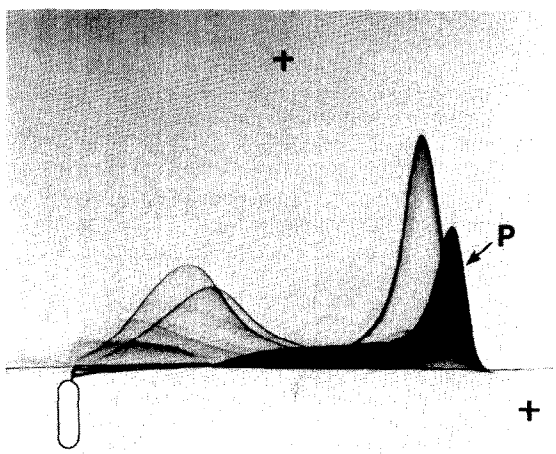

Fig.1. Crossed immunoelectrophoresis of crude pullulanase. Dimension: 10 × 10 cm glass plate. Antigen: 30 μl crude pullulanase (17.3 U/ml). First-dimension electrophoresis: 105 min, 6.4 V/cm, 8°C, in 1% agarose in barbital buffer, pH 8.6, ionic strength 0.02. Anode to the right. Antibodies: 1.5 ml against crude pullulanase ~ 18.6 μl/cm² gel area. Second-dimension electrophoresis: overnight, 2.0 V/cm, 8°C. Anode at the top. Staining: Coomassie Brilliant Blue. P: pullulanase precipitate.

the column while pullulanase itself is apparently retained. However, examination of the eluate after only 50 ml had passed through the column showed that the component indicated by O was partially retained, which also necessitated an extensive washing procedure. Component O exhibited α-amylase activity inasmuch as it was capable of hydrolysing amylose as well as amylopectin which had been incorporated into the antibody-containing gel. Therefore the removal of this impurity is of particular importance, when pullulanase is used for structural studies of branched dextrins.

The purified pullulanase, after desorption and subsequent Sephadex G-15 column chromatography, was similarly examined using antibodies towards crude pullulanase. This is shown in fig.3 and reveals that the pullulanase had been essentially freed from its original impurities. The specific activity of the purified pullulanase was 30 U/mg protein close to that of 31 U/mg protein for crystalline pullulanase [2]. The recovery of pullulanase activity was 68%. The specific activity of the crude pullulanase adsorbed onto the affinity column was 0.43 U/mg protein. Thus, in terms of

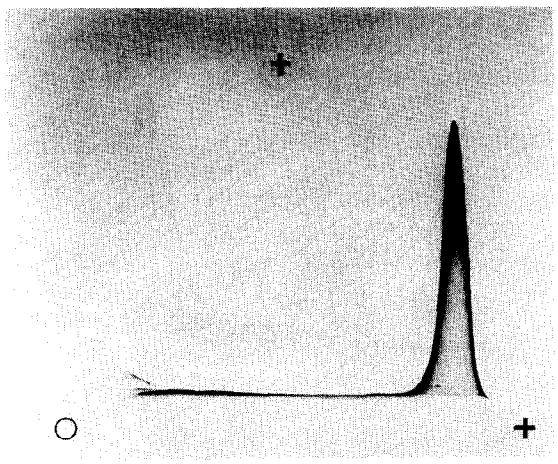

Fig.3. Crossed immunoelectrophoresis of purified pullulanase. Antigen: 10 μl pullulanase (37 U/ml) purified by biospecific affinity chromatography. First-dimension electrophoresis: 110 minutes, 6.8 V/cm, 8°C. Antibodies: 1.0 ml against crude pullulanase ~ 12.5 μl/cm² gel area. Otherwise, conditions as in fig.1.

specific activity a 70-fold purification was obtained. In addition, salts, dark-brown coloured substances and other non-proteinaceous were removed by the procedure employed.

4. Discussion

Pullulanases are produced by several species of bacteria and are also present in higher plants, where this activity original was termed R-enzyme. The classification of the plant enzyme as a pullulanase stems from the work of Taylor and Whelan [14] and Lee et al. [15] using sweet corn. Furthermore, Marshall [5] showed that sweet-corn pullulanase is similarly inhibited by Schardinger α- and β-dextrins and we have shown that plant pullulanase present in germinating barley is also inhibited by the Schardinger dextrins, the β-dextrin being the more powerful inhibitor. This indicates that the present method may similarly be useful in the isolation and purification of the debranching enzymes present in higher plants, e.g., germinating barley and barley malt.

References

[1] Wallenfels, K. and Rached, I. R. (1966) Biochem. Z. 344, 524–526.
[2] Mercier, C., Frantz, B. M. and Whelan, W. J. (1972) Eur. J. Biochem. 26, 1–9.
[3] Ueda, S. and Ohba, R. (1972) Agr. Biol. Chem. 36, 2381–2391.
[4] Ohba, R. and Ueda, S. (1973) Arg. Biol. Chem. 37, 2821–2826.
[5] Marshall, J. J. (1973) FEBS Lett. 37, 269–273.
[6] Kristiansen, T., Sundberg, L. and Porath, J. (1969) Biochim. Biophys. Acta 184, 93–98.
[7] Weeke, B. (1973) in: Quantitative Immunoelectrophoresis, (Axelsen, N. H., Krøll, J. and Weeke, B. eds) pp. 15–35 and pp. 47–56, Universitetsforlaget, Oslo.
[8] Danish Patent: DK-129141.
[9] Bender, H. and Wallesfels, K. (1966) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N.O. eds) Vol. 8, pp. 555–559, Academic Press, New York.
[10] Lowry, O. H., Rosebrough, N. J., Farr, L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Schmidt, F. and Enevoldsen, B. S. (1976) Carlsberg Res. Commun. 41, 91–110.
[12] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) Anal. Chem. 28, 350–356.
[13] Carter, J. H. and Lee, E. Y. C. (1971) Anal. Biochem. 39, 521–527.
[14] Taylor, P. M. and Whelan, W. J. (1965) Biochem. J. 95, 26P.
[15] Lee, E. Y. C., Marshall, J. J. and Whelan, W. J. (1971) Arch. Biochem. Biophys. 143, 365–374.