IMMUNOELECTROPHORETIC DETECTION OF CELLULASES

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Received 21 February 1980

1. Introduction

The utilization of cellulose as a renewable resource has created considerable research interest in cellulolytic enzymes. Several enzymes are known which cooperate in the hydrolysis of cellulose [1]. It is impossible to identify one specific cellulase component in an enzyme mixture using the conventional methods for activity determination because all individual enzymes attack the same cellulosic substrates and produce similar hydrolysis products.

Immunodiffusion methods are used in the analysis of complex protein systems because the individual protein components can be identified, separated and compared. Accurate and specific methods have been developed for the characterization of enzymes [2]. Many enzymes, e.g. glycosidases [3], retain their activity in immunoprecipitates and can be demonstrated using histochemical methods. An approach to the use of immunochemical methods for the detection of cellulases has been described [4]. The method however was based on protein estimation after immunoreaction and the specificities of the sera were not clearly demonstrated. In the method presented here for *Trichoderma reesei* cellulases the immunoreaction is followed by determination of the enzymatic activity.

2. Materials and methods

2.1. *Enzyme preparations*

Trichoderma reesei strain VTT-D-78085 was cultivated on sulphite cellulose and distiller's spent grain for 3–4 days at +29°C and pH 4.5 [5]. The proteins excreted into the cultivation medium were concentrated by means of ultrafiltration on PM 10 Amicon filters. The cellulolytic components were separated by precipitation with ammonium sulphate between 20 and 40% saturation. The precipitate was dialysed against water in Visking dialysis tubes and freeze-dried. This preparation was used to induce antibodies.

The purification was continued by adsorbing the *Trichoderma* cellulases on to amorphous cellulose at 0°C in 0.05 M sodium citrate buffer at pH 5.0. The unadsorbed material was washed out with citrate buffer and the attached enzymes released by incubating at +50°C for 20 h. The proteins obtained were further fractionated in Sephadex G-100 and G-75 columns with 0.05 M phosphate buffer at pH 7.0. Active fractions were dialysed and freeze-dried. β-Glucosidase was purified on DEAE Sephadex A-50 by eluting with 0.01 M ammonium acetate buffer at pH 5. The enzyme was then dialysed and freeze-dried.

2.2. *Preparation of antiserum*

A sample of 2.5 mg of dried cellulase preparation was dissolved in 1 ml of saline and a stable emulsion was prepared by adding 1 ml complete Freund's adjuvant. Rabbits were injected intradermally at each of 5 sites with 0.2 ml of the emulsion, giving a total dose of 2.5 mg antigen per animal. The animals were boosted at monthly intervals and blood samples were drawn from the central artery of the ear 10–12 days after each dose.

2.3. *Preparation of amorphous cellulose*

125 g Whatman CF 11 cellulose powder was suspended in 3000 ml water and the fine particles were removed by several successive decantations. Of the powder remaining, 50 g was milled in 500 ml

ethanol which a ball mill for 24 h. The powder was transferred to 500 ml 0.05 M citrate buffer, pH 5 and filtered through a Schott No. 1 fritted glass filter. The amorphous cellulose prepared had a particle size of 90–150 μm and an index of order [6] smaller than 0.5. This cellulose was used both as substrate in activity determinations and as sorbent in the purification of the enzyme preparations.

2.4. *Electrophoresis*

Immunoelectrophoresis [2] was performed on agarose gel in 0.05 M barbital buffer at pH 8.2 for 20 h with 1 V/cm. The cellulolytic activity was detected by incubating the dried immunoplates in contact with substrate plates. The substrate plates consisted of 0.5% amorphous cellulose and 1% agarose in 0.05 M citrate buffer, pH 5. Activity incubation took place in moist chambers at +50°C for 24 h. The cellulases were detected as clear zones in the turbid gels. β-Glucosidase activity was demonstrated on the dried plates using bromo-naphthyl-β-glycoside as substrate [3]. Proteins were stained with amidoblack [2].

3. Results and discussion

The antiserum contained antibodies against *Trichoderma reesei* proteins bearing β-glucosidase and cellulase activity. Antiserum diluted to 1 : 5 reacted with these proteins in *Trichoderma reesei* protein mixtures and totally inhibited their activities. Immunoelectrophoresis resolved the proteins of the crude cellulase preparation into at least nine protein arcs (fig.1). The number of protein arcs decreased during purification of the enzyme preparation. The purification will be described later. Most of the proteins moved towards the anode in the electrophoresis conditions used. Cellulolytic activity was detected as two crossed immunoarcs (fig.2). This indicates that *Trichoderma reesei* cellulases consist of at least two antigenically non-identical proteins. The shape of the more cathodic arc indicates that this cellulase occurs in two molecular sizes.

The β-glucosidase activity was associated with a protein moving towards the cathode independently of the cellulolytically active arcs. Another minor arc moving towards the anode was demonstrated in very concentrated solutions of *Trichoderma reesei* proteins (fig.3).

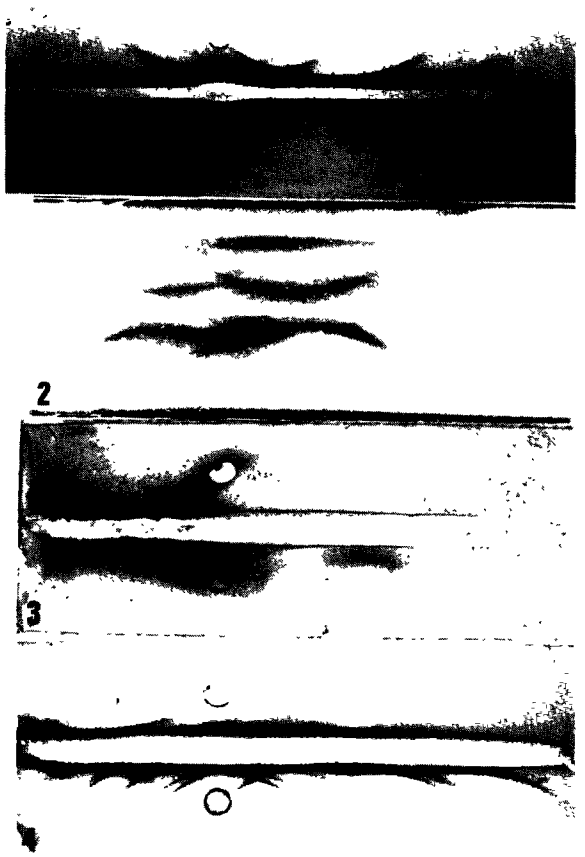

Fig.1–4. Resolution of *Trichoderma reesei* proteins in immunoelectrophoresis. (1) Protein staining: top, partially purified cellulase preparation; bottom, total protein. (2) Cellulase activity detection, the plate in fig.1; (3) β-Glucosidase activity: top, partially purified β-glucosidase preparation; bottom, total protein; (4) protein staining of the plate in Fig.3.

Purification by ion-exchange chromatography yielded a β-glucosidase preparation free of other cellulolytic activities (fig.4).

The results show that it is possible to use immunological methods for identification of the cellulases excreted by *Trichoderma reesei.* The antiserum reacted with all *Trichoderma reesei* proteins and by using the amorphous cellulose as substrate it was also possible to estimate the active cellulases.

Acknowledgements

A part of this work has been presented as a poster at 27th IUPAC Congress, 1979, Helsinki. T.-M. Enari is a research professor of the Academy of Finland.

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