ISOLATION AND PARTIAL CHARACTERIZATION OF AN EXTRACELLULAR PROTEASE FROM *SPOROTRICHUM DIMORPHOSPORUM*

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1. Introduction

The hyphae from germinated spores of wood rotting fungi are able to penetrate the wall components by liberation of solubilizing enzymes. For growth on a mixed substrate such as wood, fungi require that several enzymes be induced at the same time, the most important being β-glucanases, α-mannanases and proteases [1,2].

The production, isolation and characterization of β-1,3-glucanase by *Sporotrichum dimorphosporum* have been described [3,4] and the control of its activity after secretion reported [5]. This is the first report of the purification and some properties of an extracellular protease produced by secretion from *S. dimorphosporum* into the culture medium.

2. Materials and methods

Sporotrichum dimorphosporum was kindly provided by Dr E. T. Reese, Department of the Army (Natick MA). The organism was routinely maintained in the medium of [6] solidified with 2% agar. The organism was cultured for maximal protease production by growth in a nutrient broth containing 1% (w/v) bactopeptone, 0.3% (w/v) yeast extract and 0.2% (w/v) glucose at 30°C, stirred at 250 rev./min, for 7 days. Media were inoculated with conidia harvested from agar slants. Proteolytic activity was determined as in [7] using Azocoll as substrate. One unit was defined as the amount of enzyme necessary to give an absorbance of 1.0 under the assay conditions. The protein content of samples from the purification procedure was estimated with a modification of the method in [8]. Polyacrylamide disc gel electrophoresis was performed according to [9]. Molecular mass determination by SDS–polyacrylamide disc gel electrophoresis was performed as in [10]. Polyacrylamide gel isoelectric focusing was done as in [11]. The pH gradient was established with 1% ampholine (pH 3–10) and the protein stained according to [12].

2.1. *Enzyme purification*

The culture was centrifuged at 5000 × *g* for 1 h, the cell-free supernatant was adjusted to pH 5.5 with 1 M acetic acid.

Step 1: CM-Sepharose chromatography was performed on a 5 × 20 cm column, pre-equilibrated with 20 mM acetate buffer (pH 5.5). After loading, the column was washed with 1.2 l of buffer and the enzyme was eluted with a linear gradient of 20–600 mM acetate (pH 5.5) and with 150 ml in each reservoir. Fractions (4 ml) were collected at the rate of 25 ml . cm^{-1} . h^{-1}. The fractions were tested for activity and protein content, and absorbance was read at 280 nm.

Step 2: Enzyme containing fractions were pooled, placed in 0.25 in. d Visking tubing, concentrated to 2–3 ml by dialysis against polyethyleneglycol and then loaded on a 1.7 × 10 cm column of Sephadex G-50 equilibrated to 0.02 M acetate buffer (pH 5.5). Fractions (4 ml) were collected at the rate of 50 ml . cm^{-2} . h^{-1}, tested for activity and protein content, and absorbance was read at 280 nm.

Step 3: Affinity chromatography was performed on a 0.9 × 9.5 cm column of CBZ-D-phenylalanyl-teta–Sepharose 4B equilibrated with 0.02 M acetate buffer (pH 5.5). The enzyme solution of step 2 was applied

to the column. The column was washed with starting buffer until the absorbance at 280 nm returned to baseline. It was then eluted with 0.1 M acetate buffer (pH 3.5). Fractions of 4 ml were collected at the rate of 20 ml . cm^{-2} . h^{-1}, tested for activity and protein content, and absorbance was read at 280 nm.

2.2. *Chemicals*

All reagents were of analytical grade. Materials were obtained from the following commercial sources: Bactopeptone and yeast extract from Difco; azocoll and polyethyleneglycol from Calbiochem; CM-Sepharose, Sephadex G-50, bovine hemoglobin (type I) and bovine albumin (fraction V) were from Sigma. Ampholine (pH 3–10) from BioRad. CBZ-D-Phenylalanine-teta–Sepharose 4B from Pierce.

3. Results and discussion

The proteolytic enzyme was obtained in low yield (1.5%) with a 23-fold purification. Specific activity could not be increased further by DEAE-cellulose or exclusion chromatography. The results of purification are given in table 1. The values are those of a simple preparation but are representative of all samples. Although the purification procedure above is simple, the results of electrophoresis on denaturing polyacrylamide gel indicate a high degree of homogeneity of the protease purification. By this criterion we judge the protease preparation to be more than 95% pure. Electrophoresis in non-denaturing gels resulted in only one band.

Comparison of the relative electrophoretic mobility of the protease in SDS gels with a set of proteins of known M_r indicated a subunit of 34 000 M_r (fig.1). Gel isoelectric focusing gave a value of 7.4 for the isoelectric point and activity of the protease was

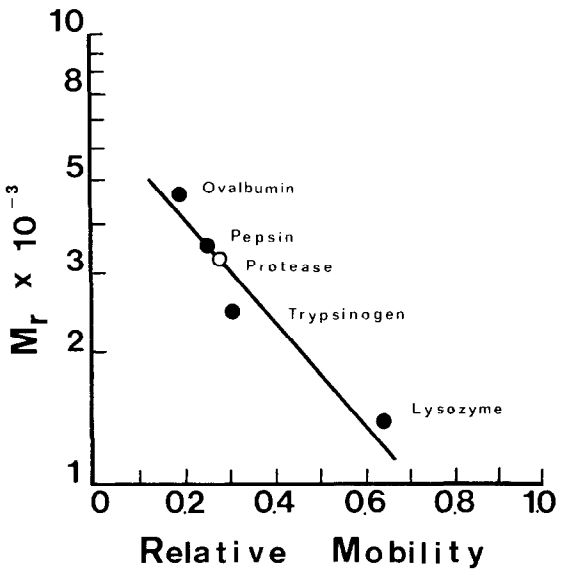

Fig.1. Determination of the subunit M_r of the purified *S. dimorphosporum* protease by electrophoresis on SDS–polyacrylamide gels. The enzyme protein and marker proteins were incubated in 1% SDS–1% mercaptoethanol for 3 min at 100°C. The samples were subjected to electrophoresis in polyacrylamide gels (7.5%) containing 0.1% SDS. The M_r of proteins were plotted on semilogarithmic scale against the distance of migration from the top of the gel, relative to that of tracking dye. The subunit M_r of each protein is: ovalbumin (45 000); pepsin (34 700); trypsinogen (24 000); lysozyme (14 300).

Table 1
Purification of *S. dimorphosporum* proteolytic enzyme

Step	Vol. (ml)	Protein (mg/ml)	Activity (total units[a])	Specific activity (unit/mg)	Yield (%)
Culture supernatant	430	1.75	709	0.94	100
CM-Sepharose	32	0.54	84.8	4.9	12
Affinity chromatography	16	0.15	10.49	21.9	1.5

[a] 1 Unit equals amount of enzyme necessary to give an A of 1.0 under the assay conditions [7]

congruent with the protein band. The protease had a pH optimum of 5.0 over pH 2.5–8.0.

This report describes for the first time an extracellular protease from a basidiomycete. Studies are being undertaken to characterize further the enzyme and find its physiological role as part of the solubilizing system for the penetration of the wall components in wood.

Acknowledgements

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