

A one-step purification of a plant lysozyme from in vitro cultures of *Rubus hispidus*

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A *Rubus hispidus* suspension culture, prepared from a callus, showed lysozyme and chitinase activities. A filtered growth medium was used as a lysozyme source. The enzyme was purified by a rapid one-step purification technique.

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1. INTRODUCTION

Since Fleming's original observations [1], few papers have been devoted to plant enzymes showing lysozyme (EC 3.2.1.17) and chitinase (EC 3.2.1.14) activities. Plant lysozymes or chitinases obtained from papaya and fig latex [2,3], turnip roots [4], wheat germs [5] and bean leaves [6] present similar enzymatic and, in part, molecular properties. Only *Rubus hispidus* callus cultures have so far been used in order to study a plant lysozyme or chitinase from in vitro cultured material [7]. In a callus, content in lysozyme and chitinase was found to be correlated to the growth rate of the culture [8]. Here, a one-step purification procedure of a lysozyme from the growth medium of homogeneous suspension cultures, obtained from callus cultures of *R. hispidus*, is described.

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2. MATERIALS AND METHODS

2.1. Callus cultures

Callus, initially obtained from cambium cells of stem explants of *R. hispidus*, were cultured in vitro at 22°C in a Heller's medium [8] solidified with 0.8% agar in white light. Glucose (50 g/l) was used as a carbon source.

2.2. Suspension cultures

40-day-old calli were suspended in a liquid modified Heller's medium. 300-ml flasks containing 100 ml of the suspension culture were incubated on an orbital shaker (150 rpm) at 24°C in the dark. After 18 days, 5 ml of the suspension was inoculated in 100 ml of fresh medium. Subculturing was performed at least 6 times.

2.3. Activity determinations

Lysozyme activity was followed by the clearance of a suspension of *Micrococcus luteus* dried cells (300 µg/ml, 20 mM formate buffer, pH 4.0). Incubation was performed at 56°C for 20 min. Hen egg white lysozyme was used as a standard [9]. Endochitinase activity was determined by the release of soluble fragments from tritiated chitin (20 mM phosphate buffer, pH 6.5). Incubation was performed at 56°C for 60 min [6,10]

2.4. Purification

A suspension culture in the growth phase was used. The suspension was filtered on a 0.45 μ m membrane (Millipore) and the growth medium concentrated on a YM-10 Amicon ultrafiltration membrane. 100 ml suspension culture was concentrated to 3 ml. The sample was then chromatographed on a DEAE A-50 Sephadex column (10 \times 1.2 cm) using a 20 mM Tris-HCl buffer, pH 9.0. Active fractions were dialysed against water and concentrated. The whole procedure was performed at room temperature. At each purification step, the protein content was determined [11].

2.5. SDS-PAGE

Laemmli's discontinuous system [12] was used (12.5% acrylamide). The samples were denatured at 90°C for 3 min in a 100 mM Tris-HCl buffer, pH 8.5, containing 1% SDS. Molecular mass standards from Pharmacia were used. Proteins were stained with Coomassie blue (Serva).

3. RESULTS AND DISCUSSION

3.1. Suspension cultures

Protein, chitinase and lysozyme activities were determined in the culture medium for different incubation times. The results are given in table 1. The protein content in the culture medium increased with the age of the culture. Lysozyme and chitinase activities were always present in the growth medium with a maximum during the active

growth phase for the lysozyme activity and during the stationary phase for the chitinase activity. In a stationary culture the viscosity of the growth medium was increased by high molecular mass polysaccharides exported by the cells. Such a medium was unsuitable for further protein purification. Growth media of 20-day-old cultures were thus used for lysozyme purification.

3.2. Purification

Published data [4–6,13,14] indicated a *pI* value for lysozymes ranging from 9 to 10.5. This high value prompted us to try the purification of the enzyme from the suspension cultures by a one-step procedure. We used a DEAE A-50 Sephadex column at pH 9.0. 100 ml filtered growth medium concentrated to 3 ml were put onto the column. The lysozyme was therefore not retained. The active fractions were pooled and dialysed against water. The results are reported in table 2. Lysozyme activity increased 3-times when compared to chitinase activity. Indeed another protein, showing a lower *pI* value, was responsible in part for the chitinase activity detected in the growth medium; this protein was lost during the present purification procedure.

The growth medium contained a population of 10 major proteins with molecular masses ranging from 19 to 88 kDa, as shown by SDS-PAGE (fig 1A and B). The purified lysozyme active fraction gave rise to a unique protein band of 32 kDa (fig 1C). This *M_r* value was similar to those

Table 1

Protein, lysozyme and chitinase contents and lysozyme vs chitinase ratio (μ g lysozyme/ 10^3 dpm) in the growth medium (100 ml) of a suspension culture as a function of time

	Incubation time (days)					
	6		18		37	
Protein (mg/ml)	0.022	\pm 0.003	0.047	\pm 0.001	0.054	\pm 0.008
Lysozyme (μ g/ml)	1.12	\pm 0.08	11.1	\pm 0.4	0.48	\pm 0.08
(μ g/mg)	25	\pm 5	118	\pm 7	4	\pm 1
Chitinase						
(10^3 dpm/ml)	3.7	\pm 0.3	7.5	\pm 0.4	22	\pm 1.8
(10^3 dpm/mg)	84	\pm 17	80	\pm 6	203	\pm 64
Lysozyme/chitinase	0.31	\pm 0.04	1.5	\pm 0.1	0.022	\pm 0.005

Table 2

Protein, lysozyme and chitinase contents and lysozyme vs chitinase ratio (μg lysozyme/ 10^3 dpm) in the filtered growth medium (A), after dialysis and concentration (B) and in active pooled and dialysed fractions (C)

	Fraction		
	A	B	C
Protein (mg)	3.66 ± 0.09	1.611 ± 0.003	0.13 ± 0.02
Lysozyme ($\mu\text{g}/\text{mg}$)	282 ± 28	130 ± 3	1090 ± 200
Chitinase (10^3 dpm/mg)	188 ± 32	44.9 ± 0.4	250 ± 48
Lysozyme/chitinase	1.5 ± 0.1	2.9 ± 0.3	4.36 ± 0.08

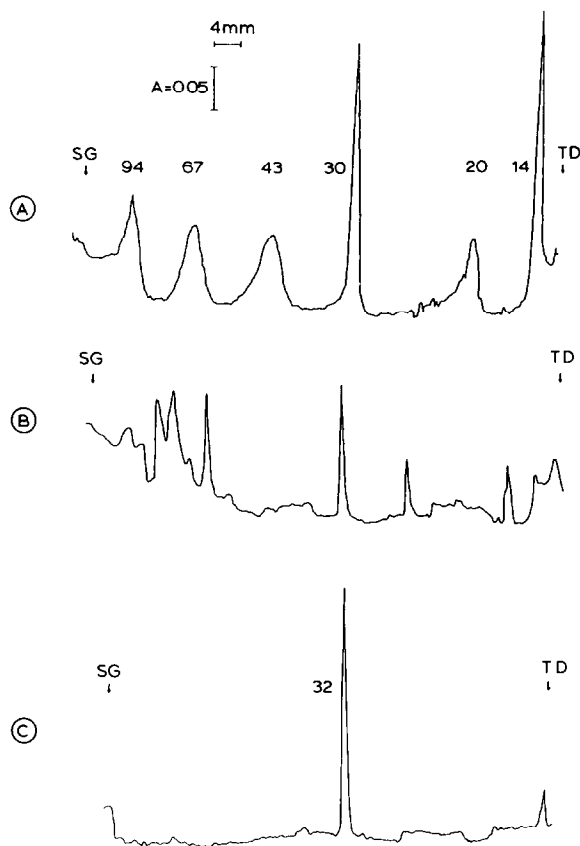


Fig 1 Scanning at 595 nm of Coomassie blue-stained polyacrylamide gels (A) Pharmacia standards; (B) growth medium and (C) purified lysozyme fraction. The molecular masses are expressed in kDa. SG, end of the stacking gel, TD, tracking dye

previously obtained with other plant lysozymes [4-6].

4. CONCLUSION

Lysozyme and chitinase activities were present in a suspension culture of *R. hispidus*. The corresponding enzymes were found in the growth medium. A lysozyme was purified by a one-step procedure from the growth medium. The purified enzyme also showed a high endochitinase activity on high- M_r chitin polymers. Such a suspension culture can thus be used as a source of a plant lysozyme and be submitted to further molecular studies.

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