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ENZYME ACTIVITIES IN EXTRACTS OF ANTHRAQUINONE-CONTAINING CELLS OF *GALIUM MOLLUGO*

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Key Word Index—*Galium mollugo*; Rubiaceae; cell culture; anthraquinones; shikimate dehydrogenase; chorismate mutase.

Abstract—Cell suspension cultures of *Galium mollugo* produce large amounts of anthraquinones which interfere with measurements of some enzyme activities. A method has been developed for determining enzyme activities in protein extracts of these cells using protamine sulfate and Servachrome XAD2. The efficiency of this method was demonstrated by measuring the activity of an enzyme sensitive to anthraquinones, viz. chorismate mutase (EC 5.4.99.5).

INTRODUCTION

Although quinones are biosynthetically derived from a wide variety of different precursors [1] they belong to the small group of secondary metabolites that are produced with high yields in plant cell suspension and callus cultures [2–7]. Different components of the media have been shown to influence the biosynthesis of quinones in cultures. Among these components are biosynthetic precursors [2, 4], nutritional factors [2, 4, 5], plant growth regulators [2, 4–7] and amino acids [2], in particular tryptophan which inhibits the synthesis of anthraquinones from its precursors by 50% at a concentration of 10^{-5} M [8].

Investigations of these regulatory phenomena have been hampered, however, by the fact that no enzyme involved in the biosynthesis of anthraquinones has been characterized. Progress has recently been made in the cell-free synthesis of precursors of bacterial quinones [9]. Isolation of the respective enzymes from higher plants is, therefore, desirable but seems to pose great problems because anthraquinones occur predominantly in lignified tissue of the root bark [2]. Cells cultivated in suspensions may, therefore, represent a more suitable enzyme source.

Microscopic examination of *Galium mollugo* cells growing in liquid media [4] reveals that anthraquinones are located in the vacuole. They are spatially separated from the bulk of the protein but homogenization of the cells inevitably results in a mixture of anthraquinones and enzymes with the latter being inactivated by the quinones [10].

We have, therefore, developed a method for immediately trapping quinones during homogenization. The efficiency of this method has been demonstrated by measuring the activity of chorismate mutase (EC 5.4.99.5), an enzyme which is rapidly inactivated in the presence of anthraquinones.

RESULTS AND DISCUSSION

Preparation of enzyme extract free of anthraquinone

The experiments were carried out with two lines of *Galium mollugo* cells. One of these strains, the coloured culture, produces anthraquinones, lucidin primveroside being the main pigment [4]. The second strain, a white culture, has been selected for non-production of pigments and thus is white in appearance. Both strains were cultivated in liquid medium 'A' [4]. Protein extracts of the anthraquinone-producing culture were variously treated in order to remove anthraquinones. The success of these experiments was monitored by measuring the anthraquinone content of the protein solutions spectrophotometrically at 410 nm. Simultaneously, protein was determined before and after each experiment. Since it is known that results of protein determination can be severely affected by phenolic compounds [11], it was to be expected that anthraquinones would interfere. Different methods normally used in protein determinations were tested with the extracts. From a white suspension culture, cells were harvested and a protein extract prepared (see Experimental). To one part of this protein solution lucidin primveroside was added at a concentration matching the amount of anthraquinones in a crude extract of the coloured culture. Protein was now determined with both anthraquinone-free and the anthraquinone-containing solutions using the methods of Lowry [12], Bradford [13], Kunitz [14] and the Biuret method [15]. With the methods of Lowry, Bradford and Kunitz no difference in the amount of protein in both solutions was observed. With the Biuret method, however, a significant difference in the apparent protein concentration of both solutions was found indicating that this method was unsuitable.

This result was confirmed by extracting protein from equal amounts of cells of the white and the coloured culture. When protein of both solutions was determined with the Biuret method the anthraquinone-containing extract gave large values when compared to the anthraquinone-free extract. Since this was also true for the method according to Kunitz (Table 1), protein was determined in subsequent experiments using either Bradford's [13] or Lowry's [12] method. In some of the later experiments protamine sulfate was added to protein extracts. Therefore, the influence of protamine sulfate on protein determinations was also checked (Table 2). It was found that Bradford's method turned out to be unsuitable

Table 1. Apparent protein concentrations (mg/ml) of extracts of the white and the coloured cultures as determined by different methods

Method	White culture	Coloured culture
Bradford [13]	1.72	1.72
Lowry [12]	2.48	2.34
Biuret [15]	1.84	4.98
Kunitz [14]	1.92	4.45

Table 2. Apparent protein concentration (mg/ml) of a protamine sulfate solution (0.1 %) as determined by different methods

Method	Apparent concentration (mg/ml)
Bradford [13]	3.50
Lowry [12]	0.83
Biuret [15]	1.38
Kunitz [14]	7.47

Bovine serum albumin was used as a standard. Protamine sulfate concentration, 1 mg/ml.

in this case and hence Lowry's method was employed with protamine sulfate-containing solutions.

Separation of anthraquinones from protein was insufficient when a Amicon CF 25 Centriflo membrane cone or a Sephadex G-25 column were employed. Centrifugation of protein extracts through Sephadex G-25 [16] was also unsuccessful even when borate buffer [11] instead of phosphate buffer was used. The same observation was made when extracts of acetone powders were prepared.

Removal of anthraquinones was also attempted using Polyclar AT and various resins either alone or in combination (Fig. 1). It is clear that Servachrome XAD 2 removes a large amount of anthraquinones with relatively little loss of protein. Increasing the molarity of the buffer (0.025, 0.05, 0.1 and 0.25 M) did not improve the yield in protein. It is known [11] that quinones may bind covalently to protein, a process that should be inhibited by reducing agents [11, 17, 18]. Treatment of the protein extract of the coloured culture with Servachrome XAD 2 in the presence of dithiothreitol (0.2 mM), sodium metabisulfite (2 mM) and diethyldithiocarbamate (10 mM), however, led to no improvement of the method.

Loomis [11] has pointed out that addition of protamine sulfate to crude extracts and subsequent centrifugation will remove phenolics. When a protamine sulfate solution (2 %) was added to crude extracts of the coloured culture (final concentrations of 0.02, 0.05, 0.1 and 0.2 %) and the solution was centrifuged, up to 80 % of the anthraquinones were removed. Since it was possible (Fig. 2) that during protamine sulfate treatment some, or all, of the enzymes of interest could have been removed we also followed the activity of dehydroshikimate reductase (EC 1.1.1.25) during this treatment. It was found that the activity of this enzyme was essentially unaffected even at a final protamine sulfate concentration of 0.2 % (Fig. 2).

Since Servachrome XAD 2 and protamine sulfate proved to be the most efficient agents for removal of anthraquinones, they were now combined. A crude extract was treated with protamine sulfate (0.1 % final concentration) followed by increasing amounts of Servachrome XAD 2 (Fig. 3) and a crystal clear solution was obtained. This method enabled us to obtain protein solutions free of anthraquinones and to determine their protein content reliably.

Measurement of the chorismate mutase in extracts of the white and the coloured cells

The next question to be investigated was whether or not activity of enzymes in this extract was still detectable. Dehydroshikimate reductase was found to be rather insensitive to anthraquinones and, thus, proved to be

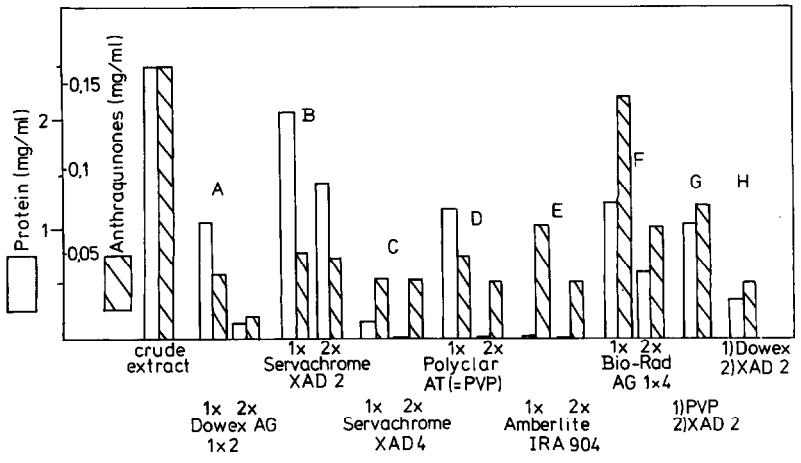

Fig. 1. Treatment of a crude protein extract of the coloured culture with various resins and Polyclar AT (PVP). In experiments A–F the first treatment (1 ×) was repeated (2 ×). In experiments G and H two different agents were used successively. Abbreviations: PVP, polyvinylpyrrolidone (i.e. Polyclar AT); Dowex, Dowex AG 1 × 2; XAD 2, Servachrome XAD 2.

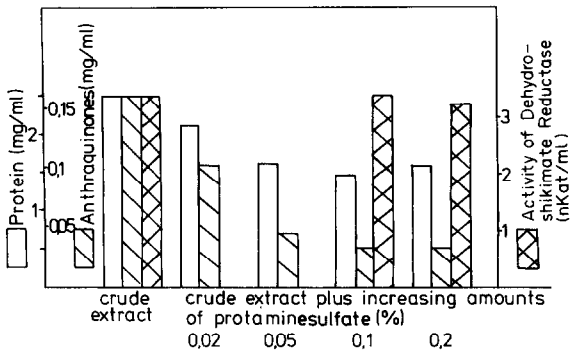

Fig. 2. Treatment of a crude protein extract of the coloured culture with increasing amounts of protamine sulfate.

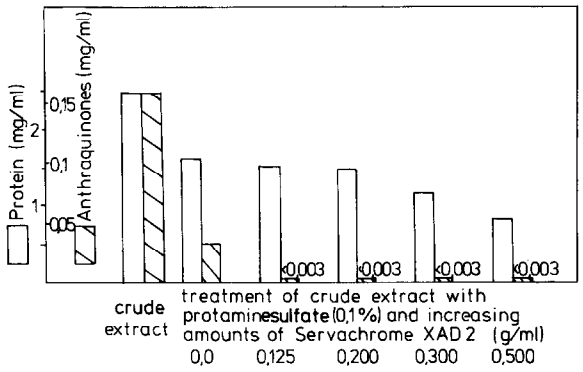

Fig. 3. Treatment of a crude protein extract of the coloured culture with protamine sulfate (0.1 %) and increasing amounts of Servachrome XAD 2.

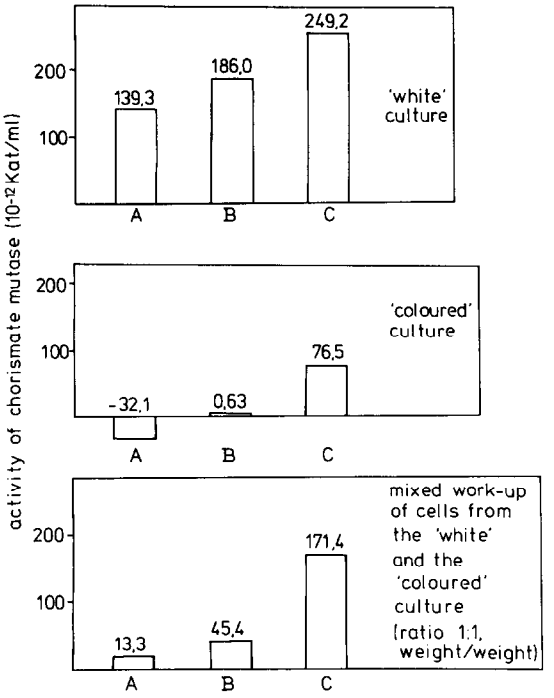

Fig. 4. Measurement of the activity of chorismate mutase in extracts of the white culture, the coloured culture and a mixture of white and coloured cells. In experiment A the extract was not treated with any agents, in experiment B the extract was treated with protamine sulfate and Servachrome XAD 2, in experiment C the cells were suspended in a buffer solution containing protamine sulfate and then cells were ruptured by sonication. Subsequently, another portion of protamine sulfate and Servachrome XAD 2 was added. In each case 5 g cells were worked-up as described in the Experimental.

unsuitable for our purpose. Chorismate mutase activity, however, was detectable in untreated crude extracts of the white culture but not in extracts of the anthraquinone-containing cells (Fig. 4, experiment A). Chorismate mutase activity in the extract of the coloured culture actually gave a negative value because the non-enzymic conversion of chorismate to prephenate was also reduced in these extracts relative to the reference. When equal amounts of white and coloured cells were mixed and a crude enzyme extract prepared, chorismate mutase activity was lower than would have been expected from the white cells alone. It, therefore, appears that chorismate mutase of the white cells was inactivated by anthraquinones from the coloured cells during extraction of protein.

When activity of the chorismate mutase was determined in the white culture, after treatment of the crude enzyme extract with protamine sulfate and Servachrome XAD 2, the activity of the enzyme was enhanced. This may be due to removal of phenolics and residual amounts of anthraquinones from the extract (Fig. 4, experiment B). When cells of the coloured strain were extracted and the extract treated with both reagents, again no enzyme activity was detectable suggesting that during extraction the chorismate mutase was inactivated. This was confirmed by extraction of mixed white and coloured cells. Again the chorismate mutase activity was less than would have been expected from the white cells alone. In a third attempt (Fig. 4, experiment C), white, coloured, and a mixture of white and coloured cells were suspended in a buffer solution containing protamine sulfate. In each case the cells were then homogenized and centrifuged. The supernatants were now treated with additional protamine sulfate followed by Servachrome XAD 2. Chorismate mutase activity in the white cells was again enhanced and activity was clearly detectable in the coloured cells for the first time. In the mixed incubation, the average activity calculated from the incubations with the white and coloured cultures was observed.

These experiments show that active preparations of enzymes sensitive to anthraquinones can be obtained. This method has also been successfully employed in the detection of *o*-succinylbenzoic acid: coenzyme A ligase, an enzyme involved an anthraquinone biosynthesis [21].

EXPERIMENTAL

Plants. Seeds of *Galium mollugo* L. were obtained from the Botanical Garden, Tübingen, W. Germany. The plants were cultivated in the greenhouse.

Callus initiation and preparation of suspension cultures. Shoots of *Galium mollugo* L. plants were sterilized, cut into pieces and transferred to agar medium 'A' [4]. In some instances the callus formed turned out to be heterogeneous. White cell lumps were picked out and transferred. Selection of white cell lumps during

repeated transfers gave a white callus which was used to start a white cell suspension culture in liquid medium 'A' [4]. Establishment of an anthraquinone-producing cell line of *Galium mollugo* has been described previously [4]. This cell line (coloured culture) was also maintained in liquid medium 'A' [4]. 300 ml flasks with 60 ml medium were incubated on a gyratory shaker (120 rpm, 28°, 400 lx). The suspension was used for further subculturing while in the early stationary phase.

Attempts to prepare anthraquinone-free protein solutions. Cells (5 g) of the coloured culture were suspended in KPi buffer (10 ml, 0.05 M, pH 7.4) and sonicated × 10 for 10 sec each time (Branson sonifier B-12 equipped with a microtip, output 60 W). Sonication was interrupted by intervals of 50 sec. The cells were cooled in ice. The temp. of the homogenate did not exceed 10°. The homogenate was centrifuged for 10 min at 50 000 *g*. Protein as determined by the Lowry method [12] was *ca* 2.5 mg/ml. When coloured cells (see above) were used extinction of this protein soln was *ca* 1.5 at 410 nm due to the presence of anthraquinones (1 cm light path). Anthraquinones and protein were determined before and after each attempt to remove anthraquinones with the aid of either various resins, Polyclar AT, Centriflo membranes CF 25 (Amicon) or protamine sulfate. Dowex AG 1 × 2, Servachrome XAD 2 and XAD 4, Amberlite IRA 904, Bio-Rad AG 1 × 4 or Polyclar AT were equilibrated in KPi buffer (0.05 M, pH 7.4) overnight.

Before use the buffer was removed by centrifugation (1000 *g*, 3 min) through a glass filter. 2.5 g of the respective resin was added to the crude extract (5 ml) and stirred for 10 min at 0°. Subsequently, the resins were separated from the protein by centrifugation and protein and anthraquinones were determined.

In attempts to remove anthraquinones with the aid of the Centriflo membrane filter CF 25 (Amicon), a protein soln (4 ml) was transferred into the filter and centrifuged at 1000 *g* for 15 min. Subsequently, the protein soln was brought back to the initial vol. of 4 ml and anthraquinones and protein determined.

In expts intended to remove anthraquinones with the aid of Sephadex G-25 (fine) a 9 × 40 mm column and the procedure of Kohl [16] were used. Sephadex G-25 (fine) was also used in a 9 × 250 mm column. In this case the protein soln (2 ml) was not centrifuged but passed over the column with a flow rate of 0.3 ml/min (KPi buffer, 0.05 M, pH 7.4).

When anthraquinones were removed using protamine sulfate (Figs. 2–4, expt B) the following procedure was chosen. A protein soln (KPi buffer, 0.1 M, pH 7.4) was stirred at 0° and a soln of protamine sulfate (2%) was added dropwise over 2 min with stirring at 0°. Stirring was continued for another 10 min. The ppt was centrifuged off (12 000 *g*, 5 min) and protein and anthraquinones determined.

Isolation of an enzyme preparation containing active chorismate mutase. Cells (5 g) were suspended in KPi buffer (10 ml, 0.1 M, pH 7.3) containing dithiothreitol (0.2 mM) and protamine sulfate (0.1%). The cells were ruptured by sonication (see above) and centrifuged. The supernatant was stirred at 0° and 0.5 ml protamine sulfate soln (2% protamine sulfate in KPi buffer, 0.1 M, pH 7.3) was added over 2 min. The soln was again centrifuged (5 min, 12 000 *g*). The supernatant was stirred with Servachrome XAD 2 (2 g) from 10 min at 0°. The resin had previously been equilibrated for 12 hr in KPi buffer (0.1 M, pH 7.3) containing dithiothreitol (0.2 mM). The pH of the protein soln was adjusted to 6.5 with H_3PO_4. This preparation was stable at −20° for not less than 6 weeks.

Shikimate dehydrogenase and chorismate mutase. Determined according to Sanderson [19] or Chu and Widholm [20], respectively.

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