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IMPROVED PREPARATION AND ASSAY OF CHALCONE SYNTHASE

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Key Word Index—*Petroselinum hortense*; umbelliferae; parsley; cell cultures; chalcone synthase.

Abstract—Preparation and assay of chalcone synthase in presence of sodium ascorbate and exclusion of oxygen during some steps gives improved yield and purity of 2*S*-naringenin.

INTRODUCTION

Chalcone synthase, a key enzyme in flavonoid biosynthesis, catalyses the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA to form 4,2',4',6'-tetrahydroxychalcone [1]. This enzyme has been widely used in studies on the biochemistry, molecular biology and genetics of flavonoids [2, 3]. A disadvantage of the presently used chalcone synthase preparations of varying purity is the formation of by-products that originate from the condensation of 4-coumaroyl-CoA with only one or two molecules of malonyl-CoA. In the first case the by-product is the respective benzalacetone or dihydropyrone, in the second the respective styrylpyrone [4, 5]. The amount of by-products varies with the concentration of 2-mercaptoethanol in the assay mixture [4]. This not only makes the quantitative enzyme assay difficult but also reduces the yield of 2*S*-naringenin (formed in the presence of chalcone isomerase) in preparative work and necessitates purification of the product. In connection with investigations on stabilization of oxygen-sensitive 2-oxoglutarate-dependent dioxygenases involved in flavonoid biosynthesis [6], we have now established a method which overcomes the above problems.

RESULTS AND DISCUSSION

For preparation of chalcone synthase from cell suspension cultures of parsley [7], 20 mM sodium ascorbate was added to all buffers instead of 2-mercaptoethanol throughout the work-up procedure. The presence of Dowex 1 × 2 and ascorbate and exclusion of oxygen during thawing of the frozen cells and subsequent homogenization were important in avoiding losses in enzyme activity. When the buffers were flushed with nitrogen before use, the 40–80% ammonium sulphate fraction containing chalcone synthase and chalcone isomerase could be stored in presence of 10% glycerol and 20 mmol · l^{-1} Na-ascorbate at −70° for several months without loss of enzyme activity.

Since ascorbate in presence of heavy metal ions is oxidized in solution above pH 7 [8], we used a phosphate buffer pH 6.8 for enzyme assays and for preparation of 2*S*[^{14}C]naringenin. Even at this suboptimal pH (pH optimum for chalcone synthase is about 8 [9]) the overall radiochemical yield of naringenin (2.33 GBq · $mmol^{-1}$) was 50–60% based on [2-^{14}C]malonyl-CoA and the product was 98–99% pure (Fig. 1a). Naringenin was identified as described [9] and its chiral purity demonstrated by a 95% conversion to [^{14}C]dihydrokaempferol with flavanone-3-hydroxylase from flower petals of *Petunia hybrida* [unpublished]. Specific activity of chalcone synthase was about 30×10^{-6} kat · kg^{-1} protein in such preparations. Furthermore, free malonic acid was not released from malonyl-CoA as has been observed in the presence of 2-mercaptoethanol [4, 5].

In contrast, chalcone synthase prepared by the published method at pH 7.5 in presence of 2-mercaptoethanol and without exclusion of oxygen had a much lower specific activity (about 6×10^{-6} kat · kg^{-1}) measured at pH 6.8 and the overall radiochemical yield based on [2-^{14}C]malonyl-CoA was only 15–20% with 50–60% content in 2*S*[^{14}C]naringenin (Fig. 1b).

For enzyme assays in crude extracts it is also advantageous to use ascorbate at pH 6.8 instead of 2-mercaptoethanol. However, the difference between ascorbate and 2-mercaptoethanol is not as pronounced as with the ammonium sulphate pellet because the exposure of the enzyme to the respective conditions is comparatively short. With the thiol reagent at pH 8.0 the usual mixture of products was found (Fig. 1b) [9]. The same preparations, when assayed at pH 6.8 gave more than 95% of naringenin as has been earlier demonstrated for highly purified chalcone synthase [1].

With ascorbate used as an oxidation protectant during preparation of crude extract as well as in the enzyme assay the pH of the assay mixture had little effect. At pH 8.0 the only detectable product was naringenin whereas at pH 6.8, 2–5% of the naringenin was further converted to apigenin, dihydrokaempferol and kaempferol, respectively. The formation of these products from naringenin is mediated by 2-oxoglutarate dependent dioxygenases present in extracts from parsley [6]. These reactions can be avoided by gel filtration of the enzyme extracts on Sephadex G-25 before assaying. Nevertheless the sum of flavonoid products originating from a complete chalcone synthase reaction was almost 100%. Although it is known [10] that ascorbate is oxidized at pH 8.0 in the presence of traces of heavy metal ions, the excess of ascorbate present in the assay is obviously sufficient to protect the enzyme during the short incubation time.

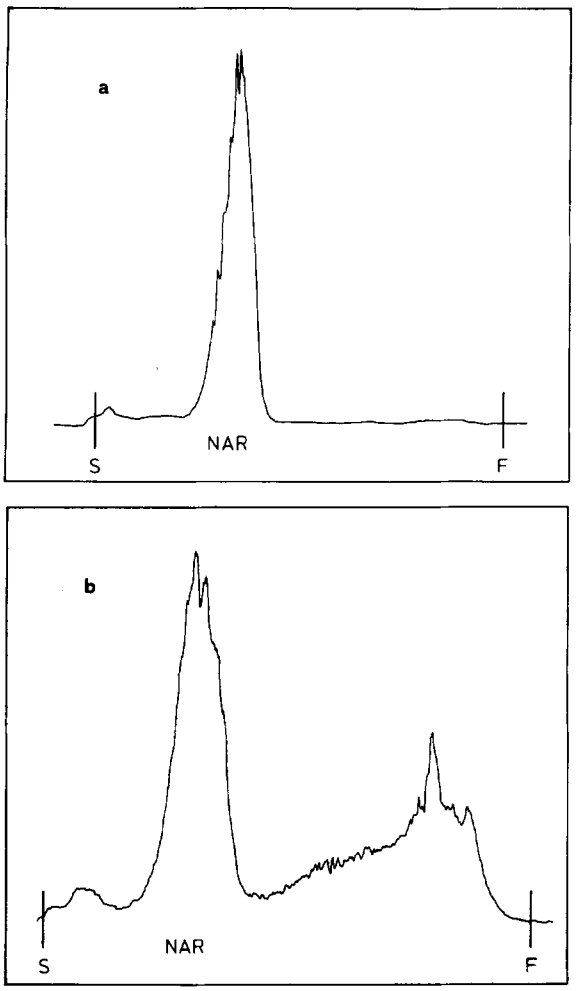

Fig. 1. (a) Radiochromatogram (cellulose, 15% acetic acid) of the ethyl acetate extract of a chalcone synthase assay utilizing an ammonium sulphate fraction (40–80%) prepared in the presence of Na-ascorbate as an antioxidant. 98.5% of the extracted radioactivity is [^{14}C]naringenin. (b) Radiochromatogram as with (a) but with the ammonium sulphate fraction prepared in presence of 14 mmol·l^{-1} 2-mercaptoethanol. Only 55% of the extracted radioactivity is naringenin. NAR, naringenin; S, start; F, front.

Isolation of enzymes from plants is usually hampered by the presence of polyphenols. Frequently anion exchangers like Dowex 1 × 2 in presence of 2-mercaptoethanol and Tris–HCl at pH 7.5 are used for removal of phenolics and to avoid oxidation reactions. However, a rapid oxidation of 2-mercaptoethanol takes place under such conditions [11], which could lead to further oxidative damage of proteins. Substitution of 2-mercaptoethanol by Na-ascorbate could therefore be advantageous not only for preparation of chalcone synthase but also for other enzymes from plants. For example, in our preparations a considerably higher activity of acetyl-CoA carboxylase was present than had previously [12] been found (data not shown).

EXPERIMENTAL

Parsley cell suspension cultures were grown as described [9]. Larger quantities were grown in 20 l air-lift fermentors [13] which were illuminated 7 days after growth of the cells in the dark. The collected cells were immediately frozen with liquid nitrogen. After 1–10 days of storage enzyme was prepared from 1–2.5 kg (wet wt) of cells as follows (amounts given for 1 kg cells).

Into a large mortar (30 cm diameter) were placed 100 g Dowex 1 × 2 (equilibrated with extraction buffer) and 8 g Na-ascorbate. The frozen cells were added and 1 l of 0.1 mol·l^{-1} Tris–HCl, pH 7.3, containing 10% glycerol and saturated with nitrogen was poured over the cells while the mixture was stirred with a large spoon. After addition of 200 g quartz sand the mixture was mortared until a temperature of 3° was reached (10–20 min). The homogenate was centrifuged at 16 000 *g* for 20 min at 5° and the supernatant was filtered through glass wool. Polyethyleneimine and ammonium sulphate precipitations were carried out as described [9, 14]. The 40–80% ammonium sulphate pellet was dissolved under N_2 in a minimum amount of 0.1 mol·l^{-1} imidazole–HCl, pH 6.8, containing 20 mmol·l^{-1} Na-ascorbate and equilibrated with N_2. After addition of 10% (v/v) glycerol the soln was frozen with liquid N_2 and stored at −70°. Enzyme assays were carried out as described [15] with 5–30 μg of protein but with a 0.1 mol·l^{-1} KPi buffer at pH 6.8 and without mercaptoethanol.

Crude extracts from small amounts of cells (1 g) were prepared as described above but with double the amount of buffer and only 2 min homogenizing at 3°. Analogous extracts were prepared with a buffer containing 14 mmol/l 2-mercaptoethanol instead of ascorbate. These crude extracts were assayed for chalcone synthase using phosphate buffers of pH 6.8 and 8.0, respectively.

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REFERENCES

1. Heller, W. and Hahlbrock, K. (1980) *Arch. Biochem. Biophys.* **200**, 617.
2. Ebel, J. and Hahlbrock, K. (1982) in *The Flavonoids: Advances in Research* (Harborne, J. B. and Mabry, T. J., eds) pp 641–679. Chapman & Hall, London.
3. Grisebach, H. (1985) in *Biosynthesis and Biodegradation of Wood Components*, pp 291–324. Academic Press, New York.
4. Kreuzaler, F. and Hahlbrock, K. (1975) *Arch. Biochem. Biophys.* **169**, 84.
5. Hrazdina, G., Kreuzaler, F., Hahlbrock, K. and Grisebach, H. (1976) *Arch. Biochem. Biophys.* **175**, 392.
6. Britsch, L., Heller, W. and Grisebach, H. (1981) *Z. Naturforsch.* **36c**, 742.
7. Kreuzaler, F., Ragg, H., Heller, W., Tesch, R., Witt, I., Hammer, D. and Hahlbrock, K. (1979) *Eur. J. Biochem.* **99**, 89.
8. Rose, R. C. and Nahrwold, D. L. (1982) *Analyt. Biochem.* **123** 389.
9. Kreuzaler, F. and Hahlbrock, K. (1975) *Eur. J. Biochem.* **56**, 205.
10. Kalus, W. H., Filby W. G. and Münzer R. (1982) *Z. Naturforsch.* **37c**, 40.
11. Koundal, K. R., Sawhney, S. K. and Sinha, S. K. (1983) *Phytochemistry* **22**, 2183.
12. Egin-Bühler, B. and Ebel, J. (1983) *Eur. J. Biochem.* **133**, 335.
13. Knobloch, K.-H. and Hahlbrock, K. (1977) *Arch. Biochem. Biophys.* **184**, 237.
14. Egin-Bühler, B., Loyal, R. and Ebel, J. (1980) *Arch. Biochem. Biophys.* **203**, 90.
15. Schröder, J., Heller, W. and Hahlbrock, K. (1979) *Plant Sci. Letters* **14**, 281.