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5-(4-ACETOXY-1-BUTINYL)-2,2'-BITHIOPHENE: ACETATE ESTERASE FROM *TAGETES PATULA*

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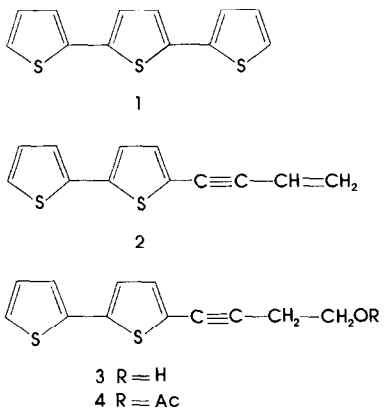
Key Word Index—*Tagetes patula*; Compositae; metabolism; acetoxybutinylbithiophene:acetate esterase; bovine serum albumin–thiophene interaction; polyacetylenes; thiophene acetylenes; acetoxybutinylbithiophene; hydroxybutinylbithiophene.

Abstract—From the aerial parts of *Tagetes patula*, an enzyme with high substrate specificity, namely 5-(4-acetoxy-1-butinyl)-2,2'-bithiophene:acetate esterase, was partly purified. The enzyme has a MW of 67000 (± 5000), pH optimum of 7.5 and its activity is affected considerably in the presence of bovine serum albumin (BSA). BSA at a concentration of 5 mg/ml in the reaction mixture prevents substrate polymerization.

INTRODUCTION

A distinct pattern of thiophene acetylenes is found in the roots and leaves of some *Tagetes* species [1, 2]. For example, α-terthiophene **1** is usually found together with 5-(but-3-en-1-inyl)-2,2'-bithiophene **2**. Similarly, 5-(4-hydroxy-1-butinyl)-2,2'-bithiophene **3** and 5-(4-acetoxy-1-butinyl)-2,2'-bithiophene **4** co-occur in a number of species [1, 2]. A number of hypotheses based on chemical relationships and on feeding experiments with labelled precursors have been proposed concerning the metabolic pathways of thiophene acetylenes, including compounds **1** and **2** [1]. The leaves of *T. patula* yield the thiophene acetylenes **1–4** along with an additional monothiophene derivative [2]. Because of this broad spectrum of relatively similar compounds, this species seems to be suitable for enzymological studies of biosynthesis or degradation. We report here the enzymatic transformation of **4** to **3** with a purified enzyme preparation from *T. patula*.



RESULTS AND DISCUSSION

This is the first report as far as we are aware, of an enzyme involved in the transformation of an acetylenic compound. Measurable enzyme activity was found after the first gel filtration step on Sephadex G-25. After four subsequent $(NH_4)_2SO_4$ precipitations and gel filtrations, hydrolase activity was eluted on Sephadex G-200 (Fig. 1). The elution behaviour of the enzyme during gel filtration is similar to that of a calibration protein with a MW of 67000 (±5000). This value is similar to the MW of subunits of carboxylic ester hydrolases from several animal and plant tissues [3–6]. However, no evidence points to the existence of such subunit aggregations in the enzyme under investigation. The time course of esterase activity shows a saturation tendency within the first minute of incubation (Fig. 2). A linear reaction course during the first 5 min of incubation could be achieved by the addition of *ca* 1 mg of BSA/ml of reaction mixture. Without BSA addition, the protein dependence shows

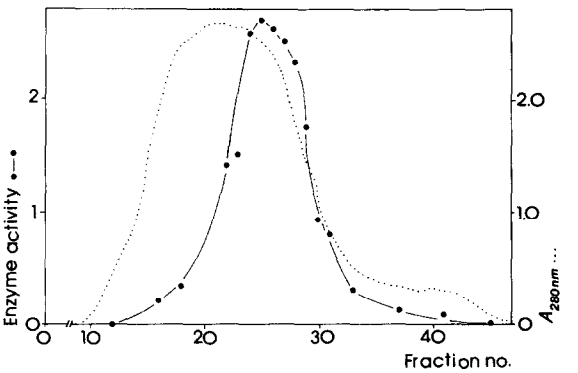


Fig. 1. Second fractionation of esterase on Sephadex G-200. Enzyme activity: arbitrary units from peak integration on HPLC (see Experimental).

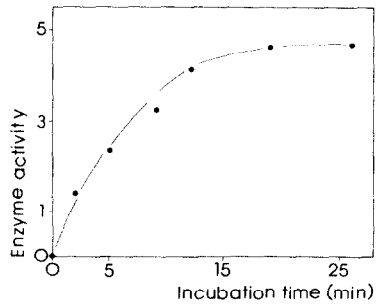


Fig. 2. Time course of esterase activity (arbitrary units, see Fig. 1).

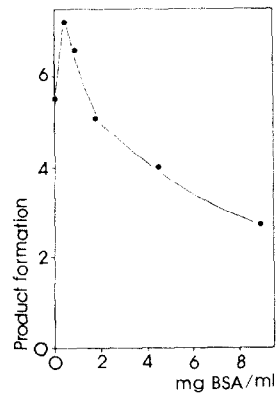


Fig. 3. Influence of BSA on product formation (arbitrary units, see Fig. 1).

a linearity up to 0.4 mg enzyme protein/ml of the reaction mixture. Also in this case, the linear range of activity could be extended by BSA addition. Using an enzyme preparation of the highest purification grade and 1 mg of BSA/ml in the enzyme assay, two different linear transformation methods [7] gave an apparent K_m value of 20 μM for the substrate at a specific activity of 26 μkat/kg protein. Without BSA addition, enzyme specific activities were lower by *ca* 30%. This low K_m value and the following investigations regarding the substrate specificity of the esterase establish 5-(4-acetoxy-1-butinyl)-2,2′-bithiophene as the true substrate of the hydrolase.

None of various acetate esters, thioacetate esters as well as other naturally occurring esters like chlorogenic acid or *p*-coumaroyl-CoA, were hydrolysed by the enzyme. This result is surprising because hydrolases, particularly esterases, have usually been reported to exhibit broad substrate specificities [3]. However, recent investigations of animal and microorganism enzymes demonstrated that some hydrolases possess exceptionally high specificities [4–6].

The purified enzyme preparation could be stored in buffer at −20° for more than three months without a marked loss of activity. After six months of repeated thawing and freezing, *ca* 50% of the esterase activity disappeared. A preparation, heated to 90° for 5 min was inactive.

As has been established for acetate esterases from other sources [3], cofactors like ascorbic acid or KCN in ranges from 10^{-6} M to 10^{-1} M or SH reagents like cysteine, glutathione, dithiothreitol, 5,5′-dithiobis-2-nitrobenzoate and 4-chloro-mercuribenzoate in ranges from 10^{-6} M to 10^{-2} M did not have any influence on the activity.

Under the assay conditions employed, 5-(4-acetoxy-1-butinyl)-2,2′-bithiophene polymerizes spontaneously at a linear rate (10% loss/5min) to give an insoluble product. On the other hand, the product 5-(4-hydroxy-1-butinyl)-2,2′-bithiophene is stable. Polymerization of the substrate could be decreased by adding BSA and prevented at a final molecular ratio of BSA–substrate of 2:1 (4 mg BSA/ml reaction mixture). However, despite a positive effect on product formation at low BSA concentration (0.5 mg/ml reaction mixture), BSA depresses esterase activity considerably (Fig. 3). It is suggested that BSA forms a weak conjugate with the substrate which inhibits the enzyme competitively. Similar results were previously reported regarding interactions between proteins and 8-methoxypsoralen [8]. Investigations concerning the nature of this particular conjugate are still in progress.

It cannot be decided whether the 5-(4-acetoxy-1-acetoxy-1-butinyl)-bithiophene:acetate esterase is involved in a degradative or a biosynthetic step of the thiophene metabolism occurring in *T. patula*. A biosynthetic pathway, beginning with **4** and yielding **1** seems to be as likely as the reverse sequence. In former considerations [1], a very different way for the synthesis of **1** and **2** was postulated. Only further enzymological investigations will elucidate these unresolved questions. The BSA–thiophene acetylene interaction arouses speculations concerning the stabilization of these light-sensitive compounds *in vivo*. Further work on this problem is in progress.

EXPERIMENTAL

Reagents and solvents. These were of the highest grade of purity available. Substrates for esterase assays were obtained commercially except for *p*-coumaroyl-CoA which was synthesized [9]. MW markers were obtained from Mann Research Laboratory (New York) and BSA from Miles Laboratory (Elkhart, Indiana).

Extraction and purification of thiophene acetylenes. All steps were performed excluding UV light and, where possible, at low temp. Whole plants of *T. patula* were ground in MeOH. H_2O was added in a ratio of 1:1, and the thiophenes were extracted into petrol, concentrated and resuspended in MeOH. After purification on Sephadex LH-20 (MeOH), thiophene acetylenes could be obtained pure by preparative reverse-phase HPLC [Micropac MCH-10 column 0.8 × 50 cm; injection valve with 1 ml sample loop; mixing 25% of 10 mM KH_2PO_4 and 10 mM tetramethylammonium chloride in H_2O (pH 3.2) and 75% of the same composition in MeCN–H_2O (4:1) (pH 3.2); flow rate 2.5 ml/min; detector $UV_{350\,nm}$; $R_{t(1-4)}$ = 67.7, 48.0, 18.5, 30.6 min]. The compounds were collected, applied to a Sephadex LH-20 column (MeOH) and analysed by UV, IR and MS. All data obtained corresponded to those previously reported [1]. For enzymological work, **4** was stored in ethyleneglycolmonomethyl ether at −20°.

Enzyme preparation. All steps were carried out at 0°. Gel filtration was performed at 4°. 300 g of aerial parts of freshly harvested *Tagetes* plants were homogenized with the addition of liquid N_2 in a mortar. After addition of an aliquot

of Polyclar AT, the powder was extracted under continuous stirring for 30 min with 0.1 M KPi buffer, pH 6.8, containing 250 mM sucrose, 1 mM dithiothreitol, 1 mM $MnCl_2$ and 50 mM K-ascorbate, pH 6.8, according to previously described extraction methods[10, 11]. Insoluble residues were removed by filtration on Whatman GFA filters and the filtrate centrifuged (28000 *g*, 15 min). Protein obtained by precipitation with solid $(NH_4)_2SO_4$ (80% satd) and by centrifugation (45000 *g*, 15 min) was redissolved in 0.1 M KPi buffer, pH 6.8. Further purification was performed by two subsequent gel filtration steps on Sephadex G-25 (column 2.5 × 30 cm), the first equilibrated with 0.1 M KPi buffer, pH 6.8, the second at pH 8. After each gel chromatography the protein-containing fractions were combined and treated with $(NH_4)_2SO_4$ (80% satd). In the same manner, two subsequent purification steps were performed using Sephadex G-200 columns (2.5 × 30 cm and 1.0 × 45 cm, respectively), equilibrated with 0.1 M KPi buffer, pH 8. The esterase-containing fractions were combined and stored at −20°. For MW estimation, the same columns were calibrated according to ref. [12].

Enzyme assay. This contained 9 nmol acetoxybutinylbithiophene in 5 μl ethyleneglycolmonomethyl ether and the enzyme preparation. BSA was added as a soln (10 mg/ml) in 0.1 M KPi buffer, pH 8. A total vol. of 300 μl was adjusted using 0.1 M KPi buffer, pH 8. The reaction was started by addition of the substrate. Incubation was performed in covered plastic tubes at 30° excluding UV light. The reaction was stopped by immediate injection of the mixture on HPLC.

Analysis of reaction mixture. After application to reverse-phase HPLC (see above; Micropac MCH-10 column 0.4 × 30 cm; mixing 10 and 90% of the solns given above; flow rate 1.0 ml/min; detector $UV_{333\,nm}$; $R_{t(3,4)} = 5.2$, 6.8 min), identification by comparison with authentic material and quantification of the compounds by peak integration was performed. Enzyme specific activity was calculated using the absorption coefficients given in ref. [13].

Protein. This was estimated by a combined Biuret and Lowry method[14] with BSA as standard.

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