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MOLECULAR WEIGHT AND AMINO ACID COMPOSITION OF PURIFIED SPINACH BEET PHENOLASE

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Abstract—The purification of spinach beet phenolase has been modified to include equilibration of the crude macerate with 0·5% cetyl-trimethylammonium bromide and chromatography on a hydroxyapatite column. Using this procedure improved yields of enzyme, homogeneous by the criterion of SDS polyacrylamide gel electrophoresis, have been obtained. The amino acid composition, molecular weight (MW) and sedimentation velocity behaviour for spinach beet phenolase are compared with corresponding data for mushroom phenolase. It is concluded that spinach beet phenolase occurs as a single species of MW 40000, under conditions when tetrameric forms of mushroom phenolase exist.

INTRODUCTION

The hydroxylation of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid (3,4-dihydroxycinnamic acid), an important step in the biosynthesis of lignin and plant flavonoids, is catalysed by a phenolase, an enzyme which has been purified from leaves of spinach beet [1,2]. In a previous study [1] the enzyme appeared to be firmly bound to particles, from which it could be partially solubilized by equilibration with $(NH_4)_2SO_4$. Subsequent studies on the distribution of spinach beet phenolase [3,4] have shown that the enzyme is mainly bound to chloroplast-lamellae.

This paper describes a modification to the purification reported previously [2] which allows a greater yield of more highly purified enzyme to be achieved. The MW, amino acid composition and sedimentation velocity properties of purified spinach beet phenolase are compared with similar data for the mushroom enzyme.

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RESULTS

Solubilization of hydroxylase activity

70% of the hydroxylation activity present in the initial macerate sedimented at 17500 *g*. No further activity was detectable in the supernatant when this precipitate was washed with homogenization buffer. In contrast, over 90% of the hydroxylation activity was recovered in the supernatant following equilibration with 0·5% (w/v) Cetavlon whereas treatment with Tween 80 (1% w/v), SDS (0·1% w/v) or Urea (8 M) liberated 27, 24 and 42%, respectively, of the recovered activity in the supernatant (Table 1). Reliable hydroxylase assays could not be obtained with Triton X-100 (1% v/v) due to solubilization of chlorophyll (causing high and variable blank values) and with deoxycholate (1% w/v) it proved impossible to obtain a clean separation of the precipitate from the supernatant fluid.

Properties of purified enzyme

Electrophoresis of 100 μg purified phenolase on SOS polyacrylamide gels showed a single band

Table 1. Solubilization of spinach beet phenolase

Treatment	Sub-fraction	*m*-units enzyme/ sub-fraction	*m*-units enzyme recovered/ treatment	Activity recovered (%)	Distribution of recovered activity (%)
None	ppt	31·9	31·9	86·0	100·0
	sup	0·0			0·0
35% Saturation $(NH_4)_2SO_4$	ppt	18·2	31·3	85·0	57·6
	sup	13·1			42·4
0·5% Cetavlon (w/v)	ppt	2·0	40·2	103·5	5·9
	sup	38·2			94·1
1% Tween 80 (v/v)	ppt	27·8	36·6	100·0	83·4
	sup	8·8			26·6
0·9% SDS (w/v)	ppt	15·2	20·0	55·0	76·3
	sup	4·8			23·7
6 M Urea	ppt	21·2	36·4	99·0	58·2
	sup	15·2			41·8

The 17500 *g* ppt obtained from initial maceration was resuspended in 0·01 M Na_2HOP_4–0·005 M citric acid buffer, pH 5·3 (500 ml containing a total of 370 *m*-units hydroxylase activity). 50 ml aliquots (37 *m*-units enzyme activity) were equilibrated with the chemicals shown in Column 1 as described in the Experimental.

of protein. The mobility of this band, relative to myoglobin, corresponded to a MW of 40000 (average of 3 runs which agreed within 5%) based on a calibration curve for proteins of known MW run under the same conditions. No appreciable absorption could be observed in the visible region of the spectrum and the E_{260}/E_{280} ratio for the purified enzyme is 1·1, with absorption maximum at 276 nm.

Solutions of phenolase in 0·1 M Na_2HPO_4–0·05 M citric acid buffer, pH 6·5, prepared by direct weighing of lyophilized hydroxyapatite fraction, gave $E_{1\,cm}^{0·1\%}$ 276 nm values of 0·367, 0·349 and 0·342 respectively. In two separate sedimentation velocity runs S_{20} apparent values of 3·45 and 3·32 were obtained from mid boundary analysis. Gs apparent analysis showed that the S_{20} apparent values of the most populous species corresponded to 3·15 and 3·0 *S* respectively.

Amino acid analysis

Four different samples of the enzyme were hydrolysed with methanesulphonic acid, as described in the Experimental, and two further samples were oxidized with performic acid and hydrolysed for 24 hr with 6 N HCl in sealed evacuated tubes. The composition of each sample, in μmol of each amino acid, was normalized to a value of arginine set equivalent to 10 residues per molecule. The normalized values for serine and threonine were plotted against time and extrapolated to zero time to give the corrected average value. Proline, isoleucine and valine may only be released slowly from some proteins, but in this case they were not found to do so.

Using this normalizing procedure it was found that all amino acids, except tyrosine, give close to integral values (Table 2). The discrepancy in this case may be due to oxidation of tryrosine residues during isolation. The consistency of the trytophan content between the different samples, together with the stability of the tryptophan value over a 120-hr hydrolysis period, provides further support for the claim [8] that hydrolysis with methanesulphonic acid provides a convenient and reliable method for determining the tryptophan content of a protein.

Summation of weights of amino acids, based on the integral values reported in Table 2, gives a MW of 35400 which is in agreement with that estimated by SDS gel electrophoresis.

DISCUSSION

Non-ionic and anionic detergents have been used by other workers to extract over 70% of phenolase from chloroplasts of apple [9], sugar beet [10] or spinach beet [4], but cationic detergents such as Cetavlon, which is particularly effective at solubilizing spinach beet phenolase without liberation of chlorophyll, have rarely been used before. Triton X-100 liberated so much

Table 2. Amino acid analysis of the purified phenolase (six samples)

	Average number of μmol ± s.d. normalized to Arg = 10	Nearest integer
Tryptophan*	2·11 ± 0·08	2
Lysine	17·83 ± 1·67	18
Histidine	7·91 ± 1·57	8
Arginine	10·0	10
Aspartic acid and asparagine	40·8 ± 5·0	41
Threonine	18·8 ± 1·76	19
Serine	23·8 ± 2·15	24
Glutamic acid glutamine	18·13 ± 1·22	18
Proline	27·86 ± 3·78	28
Alanine	22·6 ± 1·29	23
Cysteic acid†	7·2	7
Valine	26·2 ± 1·92	26
Methionine‡	7·1 ± 0·65	7
Isoleucine	14·2 ± 1·04	14
Leucine	29·66 ± 2·2	30
Tyrosine	8·31 ± 2·53	8–9
Phenylalanine	16·99 ± 2·12	17
Glycine	26·0 ± 4·37	26

* Estimated after methane sulphonic acid hydrolysis only. † Estimated after performic acid oxidation. ‡ Estimated as methionine sulphone after performic acid oxidation, or directly as methionine.

chlorophyll that previous reports [4] that this non ionic detergent solubilized up to 90% of *p*-coumaric acid hydroxylase activity from spinach beet chloroplasts could not be repeated.

With these modifications to the purification procedure it is possible to obtain, in improved yields, samples of phenolase, homogeneous by the criterion of gel electrophoresis. The $E^{0.1\%}_{276\,nm}$ of spinach beet phenolase is about 8× lower than the corresponding values (2·45–2·65) for mushroom phenolase [11]. The amino acid composition is in agreement with this as it suggests a tryptophan content of two residues/40000 *g* compared with a tryptophan content of 8 residues/32400 *g* for mushroom phenolase [12].

The MW of spinach beet phenolase appears similar to that for the *o*-diphenol oxidase from potato (36400), which also has an $E_{280/260}$ ratio of 1·1 but with absorption maximum of 267 nm [13]. In contrast it is higher than the monomer MW for mushroom phenolase (31000–33000) but lower than that of the tetramer (about 120000) [12–14]. Analysis of *Neurospora* tyrosinase by sedimentation equilibrium indicated aggregation to a tetramer at high concentration [15]. The monomer MW was 33000 ± 2000 and 8 tryptophan residues per monomer were reported. The S_{20} buffer value for spinach beet phenolase, under non-dissociating conditions is in accord with a globular protein of MW around 40000 and is in direct contrast to the $S_{20}w$ values in the range 6·7–7·2 *S* for mushroom phenolase [16]. Thus no evidence has been obtained in this study for tetrameric quaternary structure in spinach beet phenolase, such as is suggested for mushroom phenolase [16].

EXPERIMENTAL

Solubilization of hydroxylase activity. Spinach beet leaves (greenhouse grown) were macerated under the conditions in [1]. Washed leaves (100 g), from which larger veins and midribs had been removed were macerated in a Waring blender with 200 ml ice-cold 0·01 M Na_2HPO_4–0·005 M citric acid, pH 5·3, buffer. All subsequent operations were carried out as near 4° as possible. The macerate was squeezed through muslin, centrifuged at 350 *g* for 90 sec, and the ppt discarded. Supernatant was centrifuged at 17500 *g* for 15 min, the ppt suspended in homogenizing buffer (500 ml) and separate aliquots (50 ml) equilibrated with: (a) $(NH_4)_2SO_4$ (35% saturation); (b) cetyltrimethylammonium bromide (Cetavlon; 0·5% w/v); (c) Tween 80 (1% v/v); (d) Sodium dodecylsulphate (SDS 0·1% w/v); or (e) Urea (8 M). After 30 min each aliquot was centrifuged at 17500 *g* for 15 min, the ppt resuspended in homogenizing buffer (50 ml) and the hydroxylase activity of supernatants and resuspended ppts determined. Conditions of enzyme assay and definition of enzyme units are as described previously [1].

Isolation of purified enzyme. Vaughan and Butt's procedure [2] was modified to include 0·5% (w/v) cetyl-trimethylammonium bromide (Cetavlon), in Stage II (0–35% $(NH_4)_2SO_4$ saturation) and an additional step of hydroxyapatite chromatography following Stage IV (carboxymethyl cellulose chromatography). The CM-cellulose eluate was dialysed against two changes of 10^{-3} M KH_2PO_4–K_2HPO_4 buffer, pH 6·8 and 10 g hydroxyapatite was added to the dialysed soln. A 2·5 × 2·5 cm column of enzyme adsorbed to hydroxyapatite was prepared and washed with 0·2 M K_2HPO_4–KH_2PO_4 buffer, pH 6·8, until no absorbance at 280 nm could be detected in the effluent. Enzyme was eluted by a linear gradient of K_2HPO_4–KH_2PO_4 buffer, pH 6·8 up to 0·4 M. A 2- to 3-fold increase in sp. act. over the CM-cellulose stage was obtained by this modification and overall yield of hydroxylase activity was 33% of the initial macerate. Hydroxyapatite fraction was stored either as an $(NH_4)_2SO_4$ slurry, pH 7·3 or dialysed extensively against dist. H_2O and lyophilized.

Sedimentation velocity studies. Sedimentation velocity analysis was carried out on purified phenolase (2 mg/ml) dialysed extensively against 0·1 M Na_2HPO_4–0·05 M citric acid buffer, pH 6·5, in a 12-mm double sector cell, using a Beckman Model E ultracentrifuge equipped with UV optics, photoelectric scanner and temperature control. Sedimentation was carried out at 20° and 56000 rpm and was followed by measuring protein absorption at 280 nm. Apparent S_{20} buffer values were calculated either from the position of the mid point of the sedimenting boundary or from the Gs apparent distribution of the sedimenting boundary [5] from statistical treatment of data using a PDP-8 computer.

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis. Protein samples (0·2–0·6 mg/ml) were dissolved in 0·01 M Na_2HPO_4–NaH_2PO_4 buffer, pH 7·0, containing 1% SDS and 1% mercaptoethanol. Samples were incubated at 100° for 3 min and then dialysed against the suspension buffer system overnight. Up to 100 μg of sample were subjected to electrophoresis on SDS polyacrylamide gels prepared, run and stained for protein with coomassie brilliant blue [6]. Cytochrome *c* or myoglobin (horse heart) were included in all gels as an internal standard. MW of phenolase, in 1% SDS, was estimated from the linear relationship between log MW and mobility relative to myoglobin, obtained with bovine serum albumin; bovine liver catalase; egg white ovalbumin; alcohol dehydrogenase (yeast) and trypsin (bovine). Samples of lyophilized phenolase were dissolved in 6 M guanidine hydrochloride and separated from low MW contaminants by passage through a G25-Sephadex column (20 × 2 cm) equilibrated with ammonium bicarbonate (0·5% w/v). Fractions with A at 280 nm greater than 0·1 were pooled. Samples containing 0·5 mg of phenolase were lyophilized and hydrolysed at 110° for 24, 48, 72, 96 and 120 hr using a modification to the procedure of Liu [7] in which sulphonic acid is replaced by 4 N methanesulphonic acid [8]. Hydrolysates were analysed on a Jeol JLC-5A8 amino acid analyser.

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