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CONSTITUTION OF INACTIVE PHENOLASE–INHIBITOR COMPLEX IN SPINACH CHLOROPLASTS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; phenolase; inactive enzyme complex; enzyme inhibitor; volatile fatty acids; chloroplasts.

Abstract—From inactive phenolase–inhibitor complex of spinach chloroplasts, the inhibitor is liberated with ethyl acetate. The complex is re-constituted from its two components by freezing, and can be activated at elevated temperatures. The inhibitor seems to be a volatile acid, and on freezing, propionic, butyric and valeric acids can reduce the enzyme activity greatly.

INTRODUCTION

It has been known that some plant phenolase (*o*-diphenol: O_2 oxido-reductase) are present in inactive form and can be activated by detergents [1–6], proteolytic enzymes [7–9], pH change [4, 5, 10, 11], heating [4, 5], urea [4, 5, 10] as well as freezing and thawing [12]. Several interpretations suggested for the activation mechanism, including a dissociation of phenolase–inhibitor complex [1, 6], configurational (conformational) changes [1, 4, 5, 10–12], a partial denaturation [4, 5] and an association of protomeric enzymes [12].

The author has reported that the latency of spinach phenolase present in the membranes of chloroplasts is partly ascribed to its binding with a low MW inhibitor to form an inactive complex. Treatment of the enzyme in the solubilized state by acid–alkali, urea, heating as well as Triton X-100 were ineffective for activation or rather caused inactivation, although a slight activation by standing at 0°, and a remarkable, but non-reproducible enhancement by sonication have been observed [6]. The present paper deals with the activation of an inactive complex produced *in vitro*, and the probable nature of the inhibitor.

RESULTS AND DISCUSSION

Release of inhibitor and constitution of inactive complex

It has been postulated that an enzyme solution, which is prepared from acetone powder of spinach chloroplasts disrupted with Triton X-100, contains active enzyme, a low MW (diffusible) inhibitor and inactive enzyme–inhibitor complex. Reduction of phenolase activity on freezing is considered to result from formation of the complex from the active enzyme and free inhibitor [6].

Experiments were conducted to verify the presence of bound inhibitor in the form of enzyme–inhibitor complex. The enzyme solution was thoroughly extracted with ethyl acetate and the dried extract was dissolved in a volume of water equal to that of the enzyme solution originally subjected to the extraction. The enzyme solution and the extract were mixed and after standing at 0° and −20° the phenolase activity was examined. Controls were run which contained water instead of the extract (Fig. 1). Irrespective of the presence or absence of the extract, standing at 0° brought about a slight, but distinct increase of activity, and this has been ascribed to a partial dissociation of pre-existing complex which

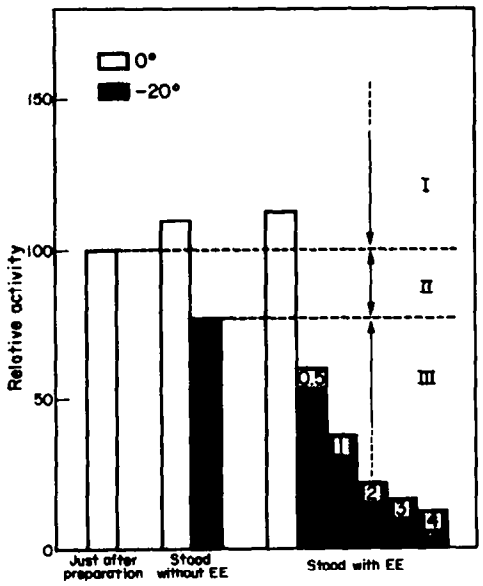

Fig. 1. Reduction of phenolase activity by freezing overnight in the presence of ethyl acetate extract (abbreviated as EE). Activity is expressed as the percentage of that of the enzyme solution just after being prepared from the chloroplast powder. Figures in the right columns represent the ratio of the extract to that of the enzyme solution.

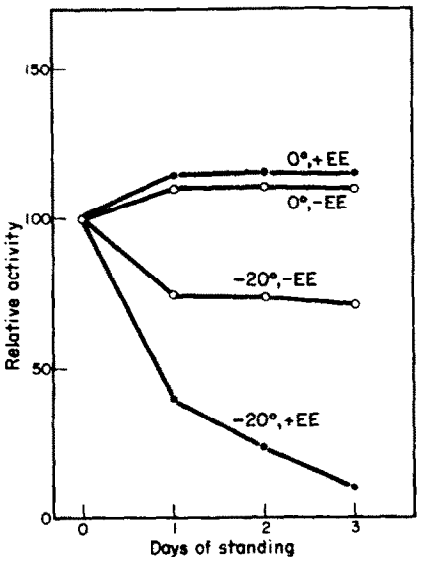

Fig. 2. Change in the activity with time at 0° and −20° (EE: ethyl acetate extract).

may correspond to I in Fig. 1. A reduction of activity *ca* 20% (II in Fig. 1) in the enzyme solution frozen without the extract is due to the formation of the complex. The activity decrease by freezing the enzyme solution with the extract is however greater than II and depends upon the amount of the extract. This is best explained by supposing that the extract contains, besides free inhibitor, an additional amount of the inhibitor which should be released from pre-existing complex by ethyl acetate (corresponding to III). Prolonged freezing was accompanied by a further reduction of activity (Fig. 2), showing

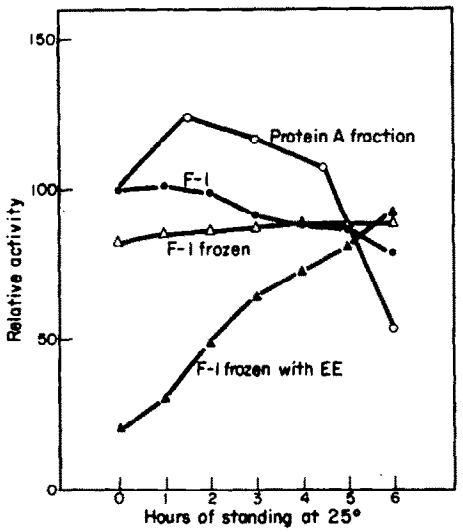

Fig. 3. Change of enzyme activity at 25° of several enzyme solutions postulated to contain the inactive complex. F-I; enzyme solution just after preparation. Protein A fraction; an eluate from Sephadex G-200 column of a solution of 40–60% $(NH_4)_2SO_4$ precipitate [6]. Freezing was carried out for two days.

that the formation of inactive complex is dependent upon the amount of the extract as well as upon freezing time. Ether also effected extraction of the inhibitor with about half the efficiency of ethyl acetate.

Reactivation of the complex

No remarkable change in activity was observed either in the freshly prepared or once-frozen enzyme solutions when stood at 25° (there correspond to F-I and F-I frozen, respectively, in Fig. 3). A partially purified enzyme solution from a Sephadex G-200 column (Protein A fraction in Fig. 3), which has been presumed to contain the active enzyme as well as the complex [6], shows first an increase, but then a progressive decrease in activity. On standing the mixture which had been frozen with ethyl acetate extract, however, a significant and successive recovery of the activity could be found at the same temperature (at 37°, first a sharp, then a declining increase of activity was observed, whilst standing at 0° brought about no or a little activation up to 6 hr incubation), and refreezing of the above mixture resulted in a clear inactivation. These facts suggest that the enzyme–inhibitor complex is produced by freezing and in turn activated at elevated temperatures presumably due to its dissociation. It seems reasonable to suppose that the association of two components is weaker in this complex than in the complex present in the chloroplasts *in situ*.

Heat activation of latent enzymes has been described for acetoacetic decarboxylase of *Clostridium acetobutylicum* [13] and a coupling factor from spinach chloroplasts [14] as well as for the nitrate reductase from *Cyanidium caldarium* [15] and *Dunaliella parva* [16]. Lorimer *et al.* have shown that the inactivation process of nitrate reductase from *Chlorella vulgaris* is associated with the firm binding to the protein of HCN [17], which is released when the enzyme is activated, e.g. by sonification [18].

Characterization of the inhibitory substances

With the chloroplasts in early October, the inhibitory substances could be released by merely treating the enzyme solution with ethyl acetate, whilst with the chloroplasts after late October, its release was achieved by heating the enzyme solution in alkaline medium (pH *ca* 10 at 95° for 20 min [6]) followed by extraction with the solvent (this was more efficient under acidic condition of pH *ca* 3–4). A highly concentrated extract was prepared from early October sample. The original enzyme solution from 1 g of chloroplast acetone powder had been dialysed against 30 vols of 0.01 M Pi buffer to remove free inhibitor and other low MW substances and the residue of exhaustive ethyl acetate extract, with a pungent odour, but without any discernible mass, was dissolved in 1 ml of water. It showed no fluorescence, and was negative to Barton's reagent for detecting phenolics [21], diazotized sulphanilic acid, ninhydrin and ammoniacal silver nitrate, while it was positive to bromocresol green. Several attempts to isolate the acidic substance(s) which might involve the phenolase inhibition have however been unsuccessful, because of the shortage of the material and its probable volatile nature.

An exploratory experiment was thus carried out with some lower MW (volatile) fatty acids in final concentrations of 1 and 2 mM buffered at pH 6.8. As compared

Table 1. Effect of some volatile fatty acids on enzyme activity

Acid	Final conc (mM)	Relative activity after standing overnight at 0°	Relative activity after standing overnight at −20°
Formic acid	2.0	99	60
Acetic acid	2.0	102	32
	1.0	107	67
Propionic acid	2.0	106	14
	1.0	103	35
Butyric acid	2.0	105	11
	1.0	103	32
	0.5		44
	0.25		65
	0.125		82
Valeric acid	2.0	103	18
	1.0	99	33
Caproic acid	2.0	109	42
	1.0	107	70
Control (water instead of acid)		100	81

The mixtures consist of 0.1 ml of enzyme solution, 0.1 ml of 0.2 M Pi buffer pH 6.8 and appropriate volumes of 5 mM fatty acids in a final volume of 0.5 ml adjusted with water.

with the control frozen without any acids, a striking reduction in activity was brought about by propionic, butyric and valeric acids, and to a lesser degree also by acetic and caproic acids, whilst a slight inhibition was given with formic acid (Table 1). The effect of longer chain fatty acids, sparingly soluble or almost insoluble in water, could not be examined, because in solutions containing water-miscible organic solvent, e.g. in 20% ethanolic solution, no freezing effect occurred even for the enzyme solution showing it distinctly, unless organic solvent was added. Standing at 0° of the above mixtures caused no remarkable change in activity (it rather increased slightly) (Table 1) and this is comparable with the results in Fig. 1. When butyric acid was used, the inhibition on freezing was parallel with its concentration from 0.5 to 2 mM, but the inhibitory effect dropped remarkably with the acid at less than 0.5 mM and no inhibition could be observed at *ca* 0.1 mM (Table 1). Unlike the complex produced from the ethyl acetate extract (natural inhibitor), no clear reactivation at elevated temperature (25°) has however been so far achieved for the mixtures frozen with inhibitory fatty acids, so that whether the inhibitor present in the chloroplasts *in situ* really is a fatty acid must await further investigations.

Natural inhibitors of plant phenolase have been suggested, such as low MW peptides in mushroom [19] and a proteinaceous substance in potato [20]. Unlike the usual inhibitors, the present inhibitor can act only by freezing in *in vitro* experiments, so that an unclarified mechanism, substituting for freezing, would operate in the 'semi-solid' membrane part of the chloroplasts.

EXPERIMENTAL

Preparation of enzyme solution from chloroplast acetone powder. Chloroplast Me_2CO powder was prepared from spinach leaves in early October as described in ref. [6]. This was suspended in 100 vol. (w/v) of 10 mM Pi buffer pH 6.8 and the suspension stirred for 1 hr. Centrifugation at 26000 *g* for 30 min gave a faint brownish supernatant, which was used as enzyme soln.

Extraction of inhibitor. Enzyme soln was shaken with an equal vol. of EtOAc 4–5 times, and the combined extracts were evapd at 40° to dryness, which was dissolved in an equal vol. of H_2O to that of the enzyme soln which had been subjected to EtOAc extraction. This aq. soln was used as EtOAc extract.

Experiments using extract. Solns in total vol. of 0.5 ml, consisting of 0.1 ml of enzyme soln, EtOAc extract (0.1 ml in standard reaction system) and H_2O, were stood at 0° in ice water or at −20° in a freezer. The control solns were added with an equal vol. of H_2O to that of the extract. Frozen samples were thawed by adding 3 ml of buffered sulphanilic acid (2 ml of 0.2 M Pi buffer pH 6.8 and 1 ml of 10 mM sulphanilic acid) which had been equilibrated at 25°, then the reaction was started, after standing for 5 min, by adding 0.5 ml of 10 mM catechol and the initial velocity was measured at 500 nm within 1–2 min. Activity measurement of non-frozen samples was also done as above.

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