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ASSOCIATION BY 2,3-DIHYDROXYBENZALDEHYDE OF MONOMERIC PHENOLASE IN SPINACH CHLOROPLASTS

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; chloroplasts; phenolase; 2,3-dihydroxybenzaldehyde (*o*-protocatechualdehyde); association; dimerization.

Abstract—In the presence of 5 mM 2,3-dihydroxybenzaldehyde, the monomeric phenolase (MW 36000) of spinach chloroplasts is completely converted to its dimer within 6 hr without significant change in activity. The aldehyde at concentrations higher than 0.25 mM could bring about this conversion after 18 hr treatment. The association of the two monomers becomes tighter with increasing concentration of the aldehyde. The dimer gave rise to a higher MW protein after freezing briefly. Several mono- and dihydroxybenzaldehydes, 2,3-dihydroxybenzoic acid, and *o*-vanillin did not produce the dimer.

INTRODUCTION

The existence of plant phenolase in multiple forms has now been established [1–3], although its occurrence as a single protein has also been found from a critical study [4]. In the chloroplasts of matured spinach leaves, two protein species with catecholase activity are present in the membrane structure [5]. The lower MW protein (A) is converted to the higher MW one (B) by concentration and the latter to the former by dilution and standing. A part of A combines with a diffusible low MW substance (X) to give rise to an inactive complex (A–X). On attempting to determine the chemical nature of X, we found that an aromatic aldehyde, 2,3-dihydroxybenzaldehyde (*o*-protocatechualdehyde), can convert A into B and a higher MW protein.

RESULTS

MW's of the two protein species

The mobility on Sephadex G-150 and -200 TLG of the two spinach enzyme species (A and B), relative to ferritin (MW 540000), corresponded to MWs of 36000 and 72000 based upon a calibration curve for proteins of known MWs (BSA 67000; eggalbumin 45000) run under the same conditions. Column chromatography on Sephadex G-150 gave the same values.

Conversion by 2,3-dihydroxybenzaldehyde of A into B and a higher MW protein

When an extract of the chloroplast acetone powder (see Experimental) was mixed with the aldehyde at a concentration of 5 mM and stood for 18 hr at 0°, the Sephadex G-150 TLG test of the browned solution revealed that A was largely converted to B. The presence of a small amount of A can be ascribed to partial reversion of B due to its dilution on the gel, because it could not be detected after electrophoresis on a cellogel plate, in which the water content is much lower than in TLG. It can thus be concluded that the aldehyde induced a complete conversion of A into the dimer, B. The above association was complete within 6 hr. When the concentration of the aldehyde is higher than 0.25 mM, only the dimer could be observed in the mixtures after standing for 18 hr (Fig. 1). The catecholase activity did not change significantly irrespective of the concentration of the aldehyde (although a slight increase and decrease were found at concentrations less than 1 mM and higher than 2 mM respectively (Fig. 1)), showing that this compound did not inhibit the enzyme, and the association occurred without masking the active site of monomeric enzyme.

It was further found that the dimer gave rise to a higher MW protein (designated as protein C), when the

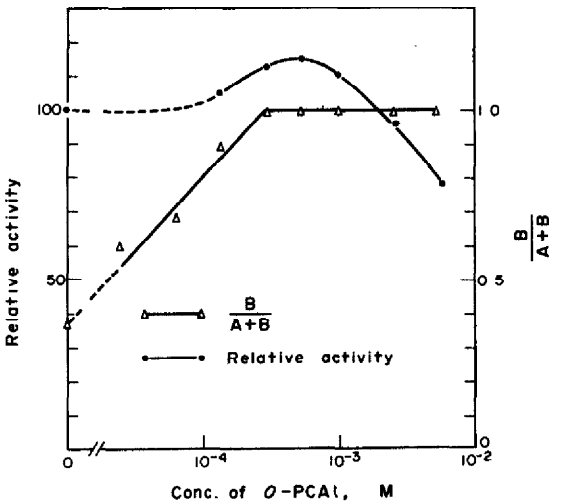

Fig. 1. The effect of varied concentration of 2,3-dihydroxybenzaldehyde on the conversion of A to B and on the activity. Analysis was carried out directly for the mixtures which had stood for 18 hr at 0°. —○—: relative activity to the control (100) and —△—; B/(A + B) × 100 and value 1.0 indicates that only B is detected.

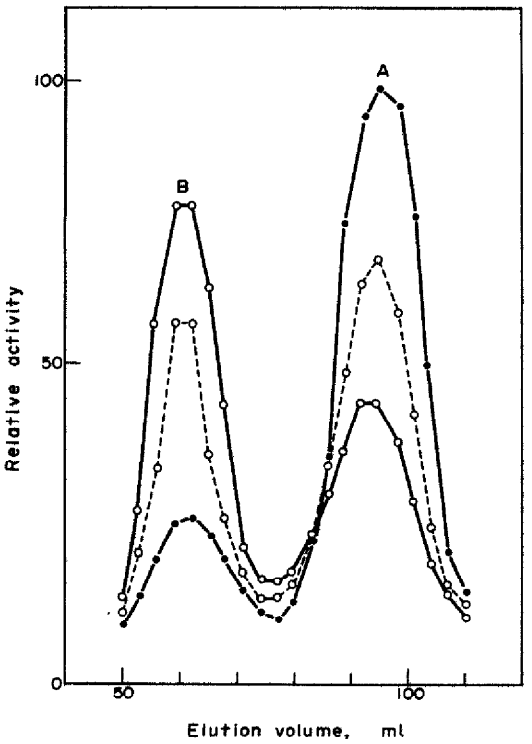

Fig. 2. Partial reversion of B into A on Sephadex G-150 column chromatography. —○— and —●—; treated with 5 and 0.5 mM 2,3-dihydroxybenzaldehyde, respectively, and upward flow started immediately after applying the sample to the column. --○--; treated with 5 mM aldehyde, and the enzyme was eluted 2 days after its application to the column. The acetone powder used in this experiment was obtained from July chloroplasts, in which only enzyme A could be detected.

mixture which contained only B, was allowed to freeze for *ca* 10 min. In contrast to the conversion from A to B, this association was accompanied by 90% decrease of activity.

The "tightness of association" in the dimer produced by the aldehyde

Standing mixtures for 18 hr containing the aldehyde at concentrations higher than 0.25 mM gave only B when examined by cellogel electrophoresis. However, as in TLG, it was found that the dimer partly reverted to the monomer, when eluted from a Sephadex column. There was less dissociation of the dimer produced by treatment with 5 mM 2,3-dihydroxybenzaldehyde than the dimer formed with 0.5 mM (Fig. 2), suggesting that the association becomes tighter with increasing concentration of the aldehyde. However, even the more stable dimer, with a tighter binding force, dissociated markedly on prolonged standing in a diluted state. Half of the dimer had dissociated when upward flow started 2 days after the enzyme was applied to the base of the column (Fig. 2). This could be due to the fact that a number of dimers occur, which differ in the degree of association.

Effects of other aromatic compounds

Several mono- and dihydroxybenzaldehydes other than 2,3-dihydroxybenzaldehyde, e.g. salicylaldehyde, 3-hydroxy- and 4-hydroxybenzaldehydes, 2,4-dihydroxybenzaldehyde, gentisylaldehyde and gentisic acid were all ineffective in forming the dimer, when mixed with the chloroplast extract at a final concentration of 5 mM followed by standing for 18 hr at 0° and examination by cellogel electrophoresis. The carboxyl-substituted 2,3-dihydroxyl compound (*o*-protocatechuic acid) as well as *o*-vanillin were also without effect, so that an aldehyde group and two OH at positions 2 and 3 seem to be necessary for the association, although it has not been determined whether the aldehyde itself, or a product derived from it by catalysis of catecholase activity, induced the dimerization. It was found that 2,5-dihydroxybenzaldehyde (gentisyl aldehyde) completely suppressed the associating action of 2,3-dihydroxybenzaldehyde, whilst none of the other substances tested showed such an inhibitory effect.

DISCUSSION

The *in vitro* interconversions of protein species with catecholase activity have been described in mushroom [7], apple peel chloroplasts [8] and wheat seedlings [2,3]. Dissociation in mushroom of a tetramer into subunits is facilitated by an elevated temperature, increased ionic strength, chemical modifications and the presence of SDS and EDTA. Multimeric phenolases are dissociated in apple and wheat with Triton X-100 and SDS or mercaptoethanol, respectively. In spinach chloroplasts, the dissociation of the dimer was brought about by mere standing as well as by dilution [6]. In the latter case this process seems to occur within a relatively short period, because the separation of proteins on a TLG plate and a Sephadex column was completed within 5 and 10 hr respectively. The dissociation of two subunit molecules seems to occur readily, and this does not contradict the results shown in Fig. 2.

On the other hand, little information is available on procedures for converting lower MW phenolase into a higher MW form. As is the case with mushroom tyrosinase, a high concentration of protein leads to polymerization [6]. Treatment with 2,3-dihydroxybenzaldehyde under appropriate conditions can result in a rapid and complete conversion of the monomer into the dimer and thereafter to a higher MW protein (C). This was not the case for mushroom tyrosinase, in which only 26 and 54% of the lower MW protein species could associate to a higher one (tetramer), respectively, 18 and 42 hr after the concentration step. It is possible that the aldehyde would be useful in obtaining multimeric proteins in a shorter time, although its effect on the multimeric forms of other plant phenolase has not yet been examined.

EXPERIMENTAL

Preparation of the Me_2CO powder of the chloroplasts, thin layer gel filtration (TLG) and the estimation of the enzyme activity have already been described [6]. 2,3-Dihydroxybenzaldehyde was synthesized from *o*-vanillin [9].

Preparation of chloroplast extract. This was obtained by suspending the chloroplast Me_2CO powder in 100 vol of 10 mM Pi buffer pH 6.8 and centrifuging the suspension, after standing for 1 hr, at 25000 *g* for 30 min.

Column chromatography. Enzyme soln used for determining the MWs of A and B by means of Sephadex G-200 column chromatography was obtained by the method already described [6]. Samples used in the experiment shown in Fig.

2 were applied as solns of the 0–20% $(NH_4)_2SO_4$ ppt from the mixtures of the chloroplast extract and 2,3-dihydroxybenzaldehyde which had stood for 18 hr at 0°. The ppt from 100 mg Me_2CO powder was dissolved in 5 ml of 10 mM Pi buffer and 3 ml fractions were collected by upward flow on a Sephadex G-150 column (2.5 × 35 cm) at flow rate of 15 ml/hr.

Electrophoresis. This was carried out on cellogel plates (5 × 18 cm) for 30 min at 10 V/cm with 10 mM Pi buffer pH 6.8. The enzymes were visualized by contacting the plate for several min with a paper sheet (Whatman 3 MM) which had been immersed in a soln containing catechol and sulphanilic acid in 0.2 M Pi buffer pH 6.8.

Determination of the ratio of A and B. After electrophoresis on a cellogel plate, zones corresponding to A and B were separately removed and the red pigment dissolved in EtOH was estimated at 500 nm.

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