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MEMBRANE ASSOCIATION AND SOME CHARACTERISTICS OF THE ETHYLENE FORMING ENZYME FROM ETIOLATED PEA SEEDLINGS[1]

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SUMMARY: When freshly prepared homogenates of etiolated pea (Pisum sativum L. cv Calvedon) subhook segments were fractionated by high speed centrifugation, the enzyme catalyzing the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene was found associated with the particulate fraction. However, on aging the homogenates at 5°C prior to fractionation, 50 to 75% of the enzyme activity partitioned into the soluble fraction; this solubilization led to a highly activated enzyme form. Both the particulate and soluble enzyme exhibited non-linear substrate saturation kinetics and were inhibited to similar extents by ascorbic acid, EDTA, $CoCl_2$ and limiting oxygen. However, they differed in their response to incubation with n-propylgallate, dithiothreitol, $CaCl_2$ and 100% oxygen. Calcium stimulated only the particulate form and increased both the 'low K_m' for ACC from 2.99 to 5.58 mM and apparent Vmax from 88 to 285 nl/mg protein/h.

Ethylene is a plant hormone regulating many aspects of growth, development and senescence in higher plants (see Ref. 1). The identification (2,3) of 1-aminocyclopropane-1-carboxylic acid (ACC) as an immediate precursor of ethylene, and in vitro demonstration (4,5) of its formation from S-adenosyl-methionine (SAM) have established the following metabolic sequence for the biosynthesis of ethylene: methionine → SAM → ACC → ethylene. However, the enzyme system that catalyzes the conversion of ACC to ethylene (ethylene forming enzyme) under physiological conditions has not unequivocally been isolated and characterized, although homogenates of pea shoots do form ethylene from ACC (6).

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In vivo, conversion of ACC to ethylene seems to be, in most cases, constitutive (7-9) and membrane-associated (10,11). We report here that the enzyme activity catalyzing the formation of ethylene from ACC may be associated with a microsomal fraction from etiolated pea subhook segments and is released (solubilized) on storage of the homogenates in the cold. Some characteristics of the membrane-associated and soluble form of the enzyme were determined. We also show that ACC saturation kinetics for both enzyme forms are non-linear and appear distinctively biphasic.

MATERIALS AND METHODS

Pea (Pisum sativum L. cv. Calvedon) seeds were imbibed in the dark for 24 h in running water. After sowing them in previously autoclaved vermiculite wetted by sterile distilled water, they were grown in the dark at 24°C for 6-7 days. The seedlings were harvested when they grew to a height of 8-11 cm.

Subhook segments, 1 cm long, were cut from the etiolated seedlings and homogenized at 2°C by grinding the material with a mortar and pestle using ice-cold 60 mM Tris-HCl buffer, pH 7.9 (0.4ml/g of tissue) (6). The homogenate was centrifuged at 11,000 x g for 15 min at 2°C. The residue was discarded. The supernatant was either taken immediately or after storage at 5°C for different periods of time, for further centrifugation at 80,000 x g for 1 h at 5°C. Both the residue (particulate fraction) and supernatant (soluble fraction) were saved. The particulate fraction was suspended carefully in 60 mM Tris-HCl buffer, pH 7.9, and the volume was adjusted to that of the soluble fraction. Protein was determined by the method of Bradford (12).

Enzymatic conversion of ACC to ethylene was carried out in 7-ml vials in a reaction mixture containing, unless otherwise stated, 100 µl of 300 mM Tris-HCl buffer, pH 7.9, 100 µl of the enzyme extract and 10 µl of ACC of a known concentration. Each reaction was run in triplicate. Control vials did not contain ACC. The vials were flushed with compressed air and stoppered with serum caps prior to incubation in a water bath at 28°C. After an appropriate incubation time, the atmosphere accumulated in the vials above the solution was sampled (2ml) and assayed for ethylene by gas chromatography (13). Each experiment was repeated at least twice. Additional methods, modifications or details are given in the text or under legends to figures and table.

RESULTS AND DISCUSSION

In preliminary experiments considerable variability was observed in the rate of enzymatic conversion of ACC to ethylene by homogenates prepared from subhook segments of etiolated pea seedlings. Subsequently, this variability in enzymatic activity was found to be related to the time of assay of the enzyme after preparation of the homogenate. Fig. 1 shows the results of an experiment where 11,000 x g supernatant was assayed immediately or after different times in storage on ice. The enzyme activity remained more or less constant

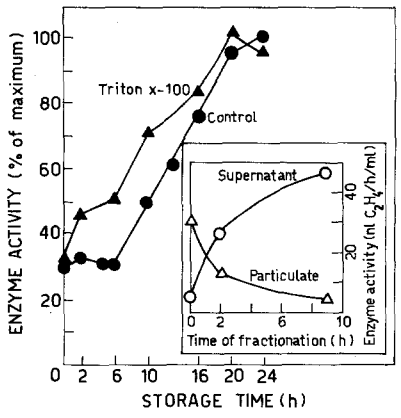

Fig. 1. Ethylene forming activity of a pea seedling homogenate stored for various times at 5°C. Aliquots of 11,000 x g supernatant (see Materials & Methods) were stored with (▲) or without (●) 1% Triton X-100 at 5°C and enzyme activity was determined at times indicated. The absolute value of the fully activated enzyme in both cases was 12.2 nl/mg/h. Inset shows change in distribution of enzyme activity as a function of storage time of the 11,000 x g supernatant incubated with 1% Triton X-100. 0, 0 - 80,000 x g supernatant; ▲, ▲ - 80,000 x g residue.

up to 6 h in storage and thereafter increased considerably (∿ 3 times), reaching a maximum at about 24 h. The lag of about 6 h in the gradual increase of enzyme activity was eliminated by addition of Triton X-100 (1% final) to the homogenate immediately after preparation and prior to storage (Fig. 1).

When a freshly obtained 11,000 x g supernatant was centrifuged at 80,000 x g for 1 h, most of the ethylene forming activity was associated with the particulate fraction (Table 1). The recovery of the enzyme activity in this fraction was ∿ 170%, suggesting the removal by centrifugation of an inhibitor from this fraction. The specific activity of the enzyme in the particulate fraction was about 16 times as much as in the soluble fraction. Similarly, distribution of enzyme activity was determined after the 11,000 x g supernatant was stored at 5°C for 20 h. As shown in Table 1 there was >50% loss in enzyme activity after high speed centrifugation of aged extracts as also observed previously (6) and the remaining activity was equally distributed between the particulate and soluble fractions. However, after aging, the specific activity of the particulate fraction decreased by about 2.5 times, while that of the soluble fraction was enriched by 4.3 times. In other experiments overnight storage resulted in 75% of the activity partitioning into the soluble fraction.

Table 1. Fractionation of the ethylene forming activity by differential centrifugation of homogenates prior to and after storage at 5°C for 20 h.

Fraction	h of storage	Total Activity (nl/h/extract) 0	20	Specific Activity (nl/h/mg protein) 0	20
11,000 x g supt.		77.50	226.80	5.86	17.16
80,000 x g supt.		12.43	53.65	1.04	4.48
80,000 x g residue		131.43	53.03	16.77	6.77

Also, overnight storage at 5°C of the 80,000 x g particulate fraction by itself did not show any increase in the enzyme activity; instead, it decreased (data not shown), suggesting that a soluble factor is responsible for releasing the enzyme from the particulate fraction. Thus, it appears that ethylene forming enzyme is associated with a membrane (particulate) fraction. Elimination of the lag period by Triton X-100 during the increase in enzyme activity on storage (see Fig. 1) was correlated with more of the enzyme activity being distributed in the soluble than in the particulate fraction (Fig. 1, inset). At 9 h in storage in buffer, the ratio of soluble(s)/particulate(p) enzyme activity was 1.19 whereas storage in 1% Triton X-100 within the same period resulted in a s/p ratio of 10.26. Thus, increase in the enzyme activity on storage of 11,000 x g supernatant is correlated with solubilization of the ethylene forming enzyme from the membrane fraction, the solubilization concomitantly leading to a highly activated enzyme form (14). Earlier, it was reported (6) that one-tenth of the extractable ethylene forming enzyme activity is associated with a particulate fraction. The discrepancy between the data of the previous report and those presented here may be due to the different pea cultivar used and/or to our use of, specifically, the 1-cm subhook segments and not entire shoots, as was done previously (6,15). The physiological significance of the release and activation following solubilization on storage of the ethylene forming enzyme from membrane is a matter for conjecture. However, its membrane association provides a potential for some control by substances which act on the membrane. Indeed, ethylene synthesis by intact tissues is disrupted by

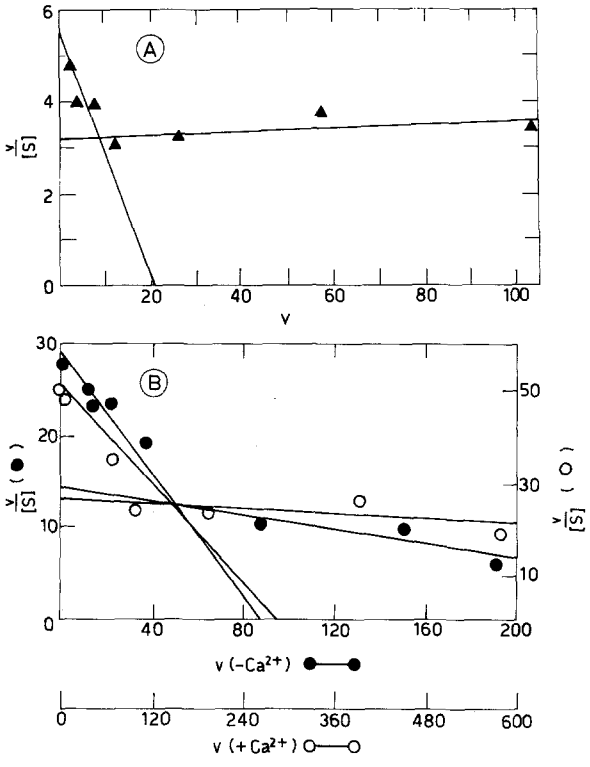

Fig. 2. v vs v/[S] plots of soluble (A) or particulate (B) ethylene forming enzyme. In B, ● minus calcium; O plus 1 mM calcium chloride. The constants obtained from linear regression analysis of the data points, separately for two distinct regions of each curve, were used to draw the lines.

treatments that interfere with membrane function (18-20), one of the metabolic sites being the conversion of ACC to ethylene (10,21).

The log-log substrate versus velocity plots (see 16) for the particulate and soluble enzyme activity showed an obvious inflection in the velocity curve for the particulate enzyme preparation while none was found for the soluble fraction, suggesting either the presence of two enzyme forms with differing affinity constants in the former preparation or the presence of complex kinetics (16). However, both enzyme preparations exhibited anomalous kinetic behaviour (Fig. 2 A,B). The exact determination of K_m values was difficult because of the gradual change in the curves. However, a lower apparent K_m for ACC was estimated to be 2.99 and 3.77 mM for the particulate and soluble enzymes, respectively. These results, in general, contrast with previous studies (6,15) which claimed that the kinetics of ACC saturation

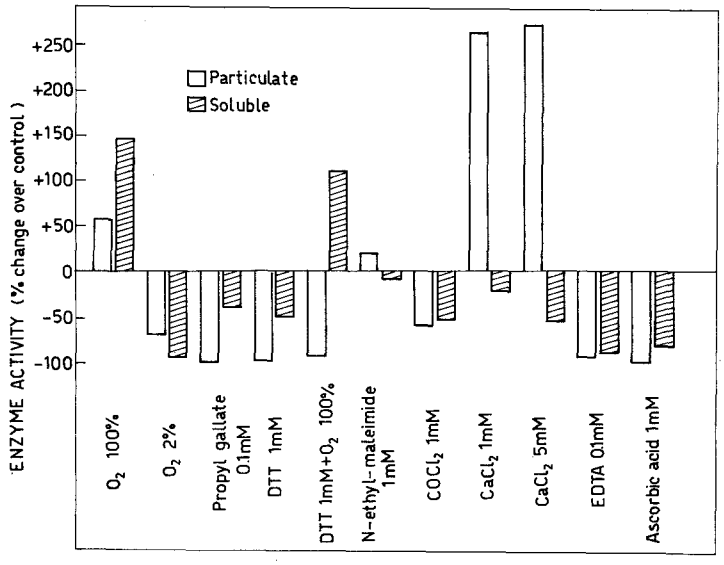

Fig. 3. Response of particulate (▣) or soluble (▨) ethylene forming enzyme to various inhibitory or stimulatory effectors.

followed classical Michaelis-Menten type. It is evident from our data that a wide ACC concentration range must be used to detect the biphasic character of the double reciprocal plots. We varied the ACC concentration up to 600-fold. The Michaelis-Menten kinetics of the enzyme reported earlier was most probably caused by working in a narrow range of ACC concentration as well as by representing the data in a Lineweaver-Burke plot, a procedure which tends to minimize any curvature in the data (16,26). Thus, the biphasic nature of substrate kinetics demonstrated here has not been recognized previously.

Ethylene forming enzyme in both membrane and soluble fractions was inhibited (Fig. 3) by oxygen deprivation (2%O_2), ascorbic acid (1 mM), EDTA (0.1 mM) and $CoCl_2$ (1 mM), known inhibitors of this reaction in vivo (2,10,17). Although the above results more or less show that the enzyme in particulate and soluble fractions share many common properties several important differences between them were also evident. The particulate enzyme was relatively more sensitive to inhibition by the free radical quencher, propylgallate, and the reducing agent, DTT. Moreover, the sensitivity to DTT of the soluble system is virtually eliminated by incubation in 100% O_2, while this was not the case for the membrane-associated enzyme. The ineffectiveness of N-ethylmaleimide

in inhibiting the enzyme suggests that inhibition due to DTT may not involve sulfhydryl groups, but may perhaps be related to involvement of free radicals in the conversion of ACC to ethylene which is supported by the inhibition of the enzyme by free radical quenchers including ascorbic acid (27) and the report of McRae et al (28). Calcium chloride (1 mM) stimulated only the particulate enzyme and increased both the 'low K_m' for ACC from 2.99 to 5.58 mM and apparent Vmax from 88 to 285 nl/mg/h. Assuming that the ethylene forming enzyme in the soluble and particulate fraction is one and the same, this observation suggests that the calcium effect on the membrane system is probably not the result of direct interaction of calcium with the ethylene forming enzyme, but may be a result of interaction of the cation with a more polar group of the membrane phospholipids (22). Further study of the calcium effect is important, particularly when calcium is known, among other effects, to stimulate ethylene production in mungbean (23) and pea (24) seedling tissue.

In addition, catalase (10 µg/ml) inhibited by 60-70% the soluble ethylene forming enzyme and had very little effect on the particulate enzyme fraction (Mattoo & Lieberman, unpublished results). Earlier it was shown (6) that catalase inhibited ethylene formation from ACC in unfractionated 11,000 x g supernatant of pea seedlings.

Finally, it should be mentioned that the major difficulty in studies of the ethylene forming system to date (6,15) is the apparently high concentration of ACC needed to saturate the enzyme. However, this may be an apparent anomaly with the enzyme systems that fulfill most, if not all, characteristics of the in vivo physiological system. Such a system may be the membrane-associated enzyme reported here. Although it showed biphasic substrate saturation kinetics, the 'low' K_m of 2.99 mM is lower by more than an order of magnitude than the 'high', almost infinite, K_m (6). Moreover, since the enzyme system used is not homogenous the presence in close proximity of other competing enzymes utilizing ACC cannot be ruled out. One such reaction could be the enzyme system that catalyzes the conjugation of ACC to malonyl-ACC, a metabolite of ACC recognized very recently (25) in higher plants.

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