Solubilization and partial purification of an enzyme converting 1-aminocyclopropane-1-carboxylic acid to ethylene in plants

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The membrane-bound enzyme capable of catalyzing the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene can be solubilized by treatment with detergent. The non-ionic detergent Nonidet P-40, at an optimum concentration of 0.2% and a protein:detergent ratio of 1:2, was found to be an effective solubilizer of the conversion activity. The solubilized enzyme resembles the membrane-bound enzyme with respect to heat sensitivity, pH dependence, Mn^{2+} stimulation and Co^{2+} inhibition. The enzyme can be further purified by means of ion-exchange chromatography with 12-fold increase in specific activity.

Ethylene *1-Aminocyclopropane-1-carboxylic acid* *Solubilization*

1. INTRODUCTION

Following the elucidation of the metabolic sequence for the biosynthesis of ethylene [1], the terminal step, conversion of ACC to ethylene, has been extensively investigated. The suggestion that the conversion reaction is mediated by a membrane-bound enzyme [2–4], prompted attempts to isolate a membrane fraction associated with the enzyme. Indeed, a microsomal fraction able to convert ACC to ethylene was isolated from various plant sources including carnation petals [5], etiolated peas [6,7] and apple fruits [8].

The microsomal system is heat denaturable, requires oxygen, is pH dependent, ACC saturable, and its activity in converting ACC to ethylene is facilitated by oxygen reactive species [6,9].

The use of the microsomal membrane system, whether representing the in situ enzyme or as a model system in the study of the mechanism of ACC to ethylene conversion, is useful. This is primarily because of its simplicity relative to the more complex plant tissue or organ. To follow this rationale attempts to isolate and purify the protein which catalyzes ACC conversion should prove useful.

Like other membrane-bound proteins which are embedded in the lipid matrix, their isolation requires solubilization procedures. This can be accomplished by use of detergents [10]. The purpose of this study was to evaluate the optimal conditions for solubilization, characterize the solubilized fraction and attempt to purify it.

2. MATERIALS AND METHODS

2.1. *Plant material*

Flowers of carnation (*Dianthus caryophyllus* L. cv. White Sim) were grown in a greenhouse, cut at a fully open stage and immediately processed.

2.2. *Isolation of membranes*

Membranes were isolated essentially as in [5].

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; Ches, *N*-cyclohexylaminoethanesulfonic acid; Epps, *N*-2-hydroxyethylpiperazinepropanesulfonic acid; Mes, *N*-morpholinoethanesulfonic acid; Tes, *N*-tris-hydroxymethylmethyl-2-aminoethanesulfonic acid

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All isolation and solubilization steps were carried out at 4°C. Flower petals were cut up finely with scissors and homogenized in a Waring blender in 10 mM Epps buffer (pH 8.5) at a ratio of 1:4 (w/v). The resulting slurry was filtered through 4 layers of cheesecloth and homogenized again in a tight-fitting Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10000 × *g* for 20 min. The resulting supernatant was centrifuged at 100000 × *g* for 1 h to yield a pellet of membranes. The pellet was resuspended in buffer and centrifuged again at 100000 × *g* for 1 h, and the resulting pellet was then resuspended in 10 mM Epps to yield the membrane fraction.

2.2. *Solubilization of membranes*

The membranes were solubilized in a solution of the non-ionic detergent Nonidet P-40. The detergent concentration, detergent:protein ratio and solubilization time were varied in the different experiments. Following detergent treatment, the mixture was centrifuged at 100000 × *g* for 1 h. The supernatant and pellet were separated and the pellet was suspended in 10 mM Epps. Protein content and ACC conversion activity were assayed in both the pellet and the supernatant. To determine the efficiency of solubilization, the amount of protein or enzyme activity in the supernatant was calculated as a percentage of the total protein or enzyme activity, respectively (i.e., in both the supernatant and the pellet).

2.4. *Protein determination*

Protein was determined by the modified Lowry method [11].

2.5. *Determination of ACC conversion activity*

The standard reaction mixture for determination of ACC conversion, unless otherwise stated, consisted of 50 mM Epps (pH 8.5), 2 mM ACC, 5 μM $MnCl_2$ and 20–50 μg membrane protein in a total volume of 1 ml. The ingredients were sealed into a 7.5 ml test tube and incubated at 30°C for 30 min, after which ethylene production was measured as in [5].

2.6. *Purification of the solubilized fraction*

The solubilized fraction was concentrated in an ultrafiltration cell (Amicon) with a cut off of M_r 30000 filter. Excess of detergent was removed with Bio-beads SM-2. The concentrated preparation was dialyzed against 10 mM Tris–HCl (pH 8.5) for 24 h with 3 changes. Then it was layered on top of a DEAE–Sepharose CL-6B column and eluted with a linear gradient of KCl. The elution buffer contained 10 mM Tris–HCl (pH 8.5) and 0.2% Nonidet P-40.

3. RESULTS AND DISCUSSION

The most useful method for solubilization of membrane components is through the action of detergents [10]. In preliminary experiments we evaluated the effect of a range of detergents, both ionic like cholic acid, sarcosine, hexadecyltrimethylammonium bromide (CATBr) and non-ionic such as Triton X-100 and Nonidet P-40, on the ACC conversion activity. The activity was preserved in the presence of non-ionic detergents while the ionic detergents abolished it. In the following experiments we employed Nonidet P-40 in evaluation of its effect on solubilization.

We first tested the effect of Nonidet P-40 on the activity itself. It was found (table 1) that the conversion activity remained unchanged in the range up to 0.09%, then rose with further increment in the concentration of the detergent, leveling off at 0.27%. Therefore, in further experiments, testing the activity following solubilization treatment, dilutions were made to lower the concentration of the detergent below 0.1%.

The results shown in table 2 indicate that the

Table 1

Effect of Nonidet P-40 on ACC conversion activity

Nonidet P-40 (%)	Conversion activity ($nl \cdot h^{-1}$)
0	26.0
0.09	24.7
0.18	52.5
0.27	59.5
0.36	58.9

Various concentrations of Nonidet P-40 were added to a reaction mixture containing 4 mM ACC, 200 μg membrane protein and 50 mM Epps (pH 8.5), and the conversion of ACC to ethylene was assayed. The values are means of two experiments, each carried out in duplicate

Table 2

Effect of detergent concentration on protein and ACC conversion activity solubilization

NP-40 (%)	Solubilization (%)	
	Protein	Activity
0	12.2	15.4
0.2	49.2	28.0
0.4	42.6	36.0
0.6	41.1	14.8
0.8	46.8	19.6
1.0	46.1	17.3

Membranes were incubated in different concentrations of Nonidet P-40 for 1 h at 4°C. Protein:detergent ratio was kept constant at 1:10 (w/w). After incubation samples were centrifuged at 100000 × *g* for 1 h and the pellet and supernatant were separated. Protein and ACC conversion activity were determined in each sample and the percentage of solubilization was calculated

detergent solubilized membrane proteins, including ACC conversion activity. Detergent concentrations of 0.2% (w/v) or higher resulted in 50% solubilization of membrane protein. The optimal detergent concentration for the solubilization of the activity was 0.2–0.4%.

The solubilization was unaffected by changing the ratio of protein:detergent in the range 1:20–1:2. Also, the duration of solubilization over the range 20–60 min did not affect the amount of solubilization.

Repeating the solubilization treatment with the remaining portion of the pellet resulted in the additional release of protein and enzyme activity into the supernatant (table 3).

Since Mn^{2+} is included in the standard reaction mixture, it is of interest to evaluate its effect on solubilization. Inclusion of Mn^{2+} in the membrane isolation and solubilization buffers had no effect on protein solubilization.

To study the effect of Ca^{2+} on solubilization, the ion was added to the isolation and solubilization buffers. It was found that in the presence of added Ca^{2+} protein solubilization was reduced. In addition, the presence of EDTA, a known chelator of Ca^{2+}, in the concentration range 0.5–2.0 mM resulted in increased solubilization, with more protein appearing in the supernatant, thus supporting the contention that the presence of Ca^{2+} may interfere with solubilization. Unfortunately, EDTA also markedly inhibited the formation of ethylene. Extended dialysis only slightly relieved the inhibition.

Table 3

Effect of repeated solubilization of the remaining pellet portion

Number of treatments with Nonidet P-40	Solubilization (%)	
	Activity	Protein
1	35.0	58.0
2	22.1	13.9
Total removed	57.1	71.9

The portion of pellet remaining after solubilization was resuspended in Epps buffer and resolubilized as before; i.e., by incubation for 1 h in the presence of 0.2% Nonidet P-40 at a protein:detergent ratio of 1:2, followed by centrifugation for 1 h at 100000 × *g*, after which the supernatant and pellet were separated. Protein and ACC conversion activity were determined and the percentage of solubilization was calculated

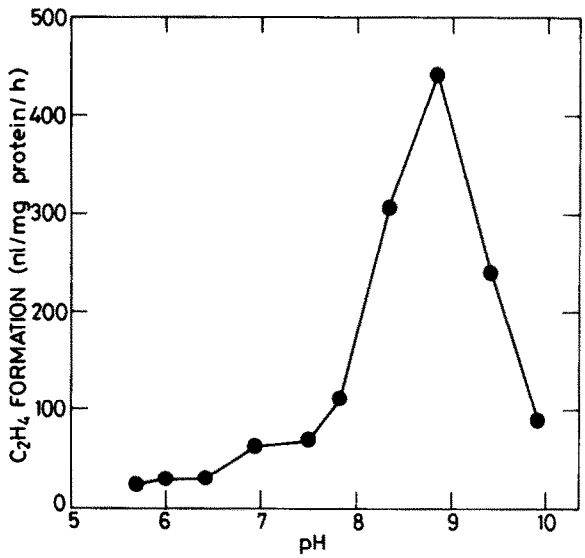

Fig.1. Effect of pH on the solubilized ACC conversion activity. The standard reaction was carried out in the presence of buffers with different pH values, and ACC conversion activity was assayed. The buffers used were: Mes (pH 5.5–6.5), Tes (pH 7.0), Epps (pH 7.5–8.5) and Ches (pH 8.75–10.0).

Similar to the membrane-bound ACC conversion activity reported in carnation petals [5], the solubilized enzyme in the present study is saturable with ACC. Its activity is pH-dependent, with an optimum at pH 8.5–8.7 (fig.1). The solubilized enzyme is heat denaturable, confirming that the solubilized membrane component is proteinaceous in nature. The preparation remains stable for at least 7 days when stored at 4°C and up to 60 days at −17°C.

Co^{2+} has been shown to inhibit specifically the step converting ACC to ethylene [12]. The solubilized enzyme is similarly inhibited by Co^{2+}, and was also promoted by manganese, as shown for the membrane-bound enzyme [13]. The results indicate a resemblance between the solubilized enzyme and that occurring naturally in plant tissues.

The solubilized enzyme was further purified on a DEAE–Sepharose CL-6B column. Upon elution with a linear gradient of KCl, an active fraction centered at fraction 8 (fig.2). The specific activity of this fraction was 12-fold higher than that of the solubilized enzyme preparation placed on the column. The activity recovered in the pooled fractions 8–12 accounts for a substantial portion of the activity placed on the column. The high specific activity was associated with only small amount of protein, well separated from other membranous proteins.

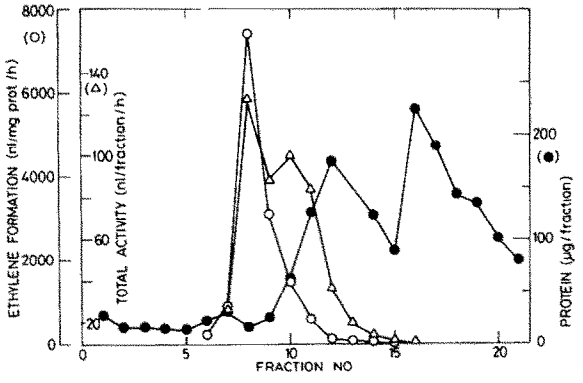

Fig.2. Elution profile from a DEAE–Sepharose CL-6B column. The solubilized enzyme was loaded on a DEAE–Sepharose CL-6B column and eluted with a linear gradient of KCl. The elution buffer contained 10 mM Tris–HCl (pH 8.5) and 0.2% Nonidet P-40. Values of protein (●), total activity (△) and specific activity (○) of the enzyme recovered from the column are presented.

In summary, this work presents a simple procedure for the solubilization of the ACC converting enzyme by mixing membrane fraction with Nonidet P-40 (0.2% w/v) at a protein:detergent ratio of 1:2 at 4°C for 20–60 min. The properties of the solubilized enzyme are similar to those of the membrane-bound enzyme. Once obtained in its solubilized form, the enzyme can be purified further by means of ion-exchange chromatography.

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