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The Extraction of ATPase from Amyloplast Envelope Membranes of Sycamore

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ABSTRACT

The objective of this study is the investigation of suitable detergents for Mg^{2+}-ATPase extraction from the amyloplast envelope membranes of suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). Among four types of detergents tested : Triton X-100, octylglucoside, sarkosinate and deoxycholate, it was found that Triton X-100 at the proportion Triton : membrane protein weight = 3:1, is suitable for ATPase-extraction. The solubilizable fraction was applied on sucrose density gradient. After centrifugation, it was found that the fraction with maximum ATPase activity possess a predominant polypeptide of 37-kDa. The result of the autoradiographic technique using adenosine triphosphopyridoxal suggested that the 37-kDa polypeptide may be a subunit of the Mg^{2+}-ATPase of amyloplast envelope membranes.

INTRODUCTION

The occurrence of a Mg^{2+}-ATPase in the chloroplast envelope membranes from spinach (Joyard and Douce, 1975), pea (McCarty and Selman, 1986a, 1986b; Nguyen *et al.*, 1987), rye (Hoinghaus *et al.*, 1985) and maize (Poincelot, 1977) has been reported. It is localized on the inner membrane of the chloroplast envelope has been established (McCarty *et al.*, 1984). Although its exact function is unknown, a possible role for this ATPase in cation (Nguyen and Siegenthaler, 1983) and protein (Pain and Blobel, 1987; Nguyen and Siegenthaler, 1983) transport into the chloroplast has been proposed.

It has been hypothesized that amyloplasts and chloroplasts are ontogenetically and evolutionarily related organelles (Bradbeer, 1981). Since amyloplasts are noncholorophyllous plastid organelle in the plant cell, one can expect that protin(s) and/or enzyme(s) localized on the inner and outer envelope membranes surrounding the amyloplast play an important role in transporting various metabolites such as carbon compounds, adenylates and protein molecule involved in the enzymic biosynthesis of starch deposited in the amyloplasts. We have characterized the ATPase of the amyloplast envelope. In addition, we have established a method for resolving the inner and outer membranes of the amyloplast in order to determine the location of the ATPase, and we have found that it is localized on the inner membrane, similar to that in green leaf chloroplasts (Harinasut *et al.*, 1988b)

It has been reported that the difference between the envelope ATPase from spinach versus pea chloroplasts is that the former is stimulated by calmodulin and Ca^{2+} (Nguyen and Siegenthaler, 1985), whereas the latter is not (McCarty and Selman, 1986a). Based on this property, Nguyen and Siegenthaler (1985) succeeded in purifying the ATPase from spinach chloroplast envelope using a Sepharose-calmodulin affinity column, while McCarty and Selman (1986a) purified the pea chloroplast enzyme by DEAE-Sephadex chromatography. The objective of this study is to investigate suitable detergents for extraction of Mg^{2+}-ATPase from the amyloplast envelope for the ultimate purpose of purification of this enzyme. In the communication, it was also suggested that the 37-kDa polypeptide on the amyloplast envelope may be the ATPase.

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MATERIALS AND METHODS

Culture : Sycamore suspension culture was subcultured every week to maintain it in the exponential phase (Harinasut *et al.*, 1988a).

Amyloplast preparation : Amyloplast was isolated from sycamore suspension culture at the exponential phase (Harinasut *et al.*, 1988a). The intactness and purity of the isolated amyloplasts were determined as previously described. (Harinasut *et al.*, 1988a).

Envelope membrane preparation : The amyloplast envelope membrane was isolated and characterized as previously described (Harinasut *et al.*, 1988a and 1988b).

ATPase extraction from amyloplast envelope : Amyloplast envelope was incubated with four types of detergents : Triton X-100, octylglucoside, sarkosinate and deoxycholate in ice for ten minutes. The suspension was centrifuged at 72,000 g for one hour. The pellet of envelope was discarded. The supernatant was determined for detergent extracted ATPase. The ATPase activity was measured as previously described (Harinasut *et al.*, 1988b).

ATP-binding protein determination : Amyloplast envelope (30 μg protein) was incubated with (^{3}H)AP$_3$-PL (50 μm). The labeled amyloplast envelope was then loaded on SDS-PAGE. ATP-binding proteins were visualized by autoradiography. In some experiments, the amyloplast envelope was preincubated with 5mM ATP.

RESULTS AND DISCUSSIONS

It was reported that amyloplast envelope membranes possessed Mg^{2+}-ATPase (Gaynor and Galston, 1983; Harinasut *et al.*, 1988a). We characterized the ATPase and illustrated its localization on the inner envelope membrane of the amyloplast (Harinasut *et al.*, 1988a and 1988b). By comparing the characteristics and location with those of chloroplast envelope membranes (McCarty *et al.*, 1984) it was suggested that they are basically the same.

Coves *et al.*, (1988) reported that Triton X-100 was the suitable detergent for extracting Mg^{2+}-ATPase of the chloroplast envelope membrane. It is valuable to find the suitable detergent to extract the ATPase for the ultimate purpose of purification and identification of the ATPase. Therefore, the suitable detergent for extraction of the ATPase from the amyloplast envelope was first determined. Figure 1 showed the effect of the detergents chosen : Triton X-100, octylglucoside, sarkosinate and deoxycholate, on Mg^{2+}-ATPase activity. The isolated amyloplast envelope was incubated with various concentrations of detergents. Then the ATPase activity associated with the amyloplast envelope was assayed. As shown in figure 1, Triton X-100 was the best among the detergents used since when the concentration was increased to 100 mM, ATPase activity still remained at 75% of the total activity (control : untreated with the detergent).

Next, suitable concentration of Triton X-100 for solubilization of ATPase associated with amyloplast envelope was determined. Figure 2 showed the correlation of Triton X-100 concentration in terms of Triton : membrane protein weight ratio and solubilization of envelope ATPase activity in terms of the difference between the total

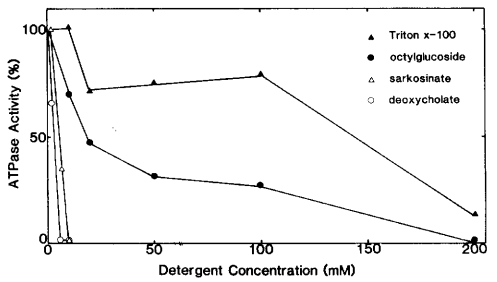


Figure 1 Effect of detergents on the activity of Mg^{2+}-ATPase. ATPase was extracted from amyloplast envelope by using various detergents Triton X-100 (▲), octyglucoside (●), sarkosinate (△) and deoxycholate (○). The ATPase activity of the extracted fraction at various detergent concentrations was measured as mentioned in the text.

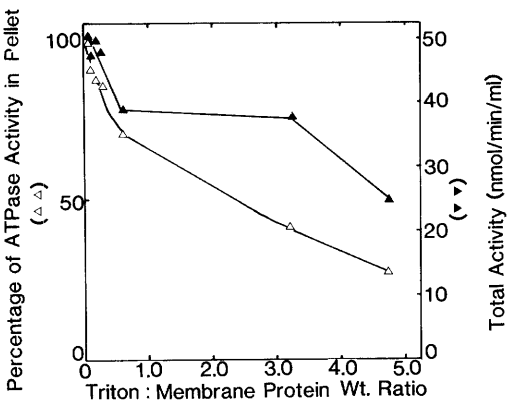


Figure 2 Solubilization of the envelope ATPase with Triton X-100. ATPase was extracted from the amyloplast envelope by Triton X-100. The ATPase activity was measured at various concentrations of Triton X-100 : membrane protein weight ratio. ▲, Total activity of envelope ATPase △, percentage of the remaining ATPase activity in the unextracted envelope-pellet.

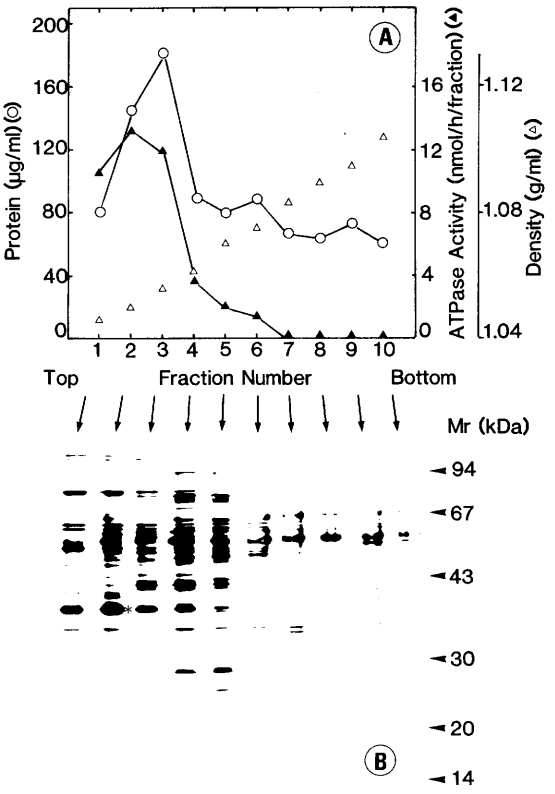
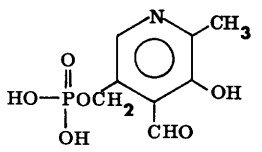


Figure 3 A. Fractionation by sucrose density gradient. The ATPase was extracted by Triton X-100 from the amyloplast envelope (Triton : membrane protein weight ratio = 3). The soluble fraction was loaded on sucrose density gradient as mentioned in the text. After 72,000g centrifugation for 14 hours, the fraction (0.5 ml) (s) were collected from the sucrose density gradient. Each fraction was determined for sucrose density (△) protein content (○), ATPase activity (▲) and polypeptide pattern as shown in figure 3B. B, Polypeptide patterns were determined on SDS-PAGE and strained with Silver-staining. (*), the 37 KDa polypeptide.

activity and ATPase activity remaining in the amyloplàst envelope. It was shown that at Triton: membrane protein weight ratio of 3, the capacity for solubilization of the envelope ATPase was about 50% while the total activity remained at 80%.

For the purpose of identification of amyloplast ATPase, the solubilization fraction carried out as shown in figure 2 was loaded on sucrose density gradient. After centrifugation, the gradient was fractionated. For further determination of the fraction enriched with ATPase activity, the ATPase activity of each fraction as well as protein content and density were assayed. As shown in figure 3A, the second fraction showed the park of ATPase activity. To determine the major polypeptide in the fraction with maximum ATPase activity, that fraction was also loaded on the SDS-PAGE. As shown in figure 3B, the predominant polypeptide possessed 37-kDa. It was reported that there are two types of ATPase associated with chloroplast envelope membrane (Nguyen and Siegenthaler, 1985; McCarty and Selman, 1986a). The activity of 37-kDa polypeptide in the chloroplast envelope of pea could not be enchanced by calmodulin, whereas the activity of 65-kDa polypeptide in the chloroplast envelope of spinach could be enchanced. We reported that the amyloplast envelope ATPase was calmodulin insensitive (Harinasut *et al.*, 1988b). Therefore, the 37-kDa polypeptide may be the amyloplast ATPase.

To confirm that the 37-kDa polypeptide is the amyloplast envelope ATPase, the affinity labelling technique was used. We reported that



Pyridoxal-5′-phosphate

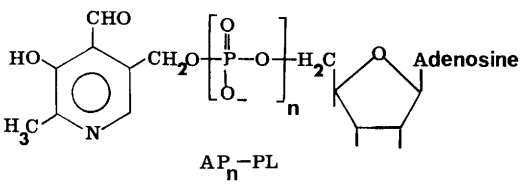


Figure 4 The chemical structure of pyridoxal 5′-phosphate and pyridoxal derivative (adenosine polyphosphopyridoxal).

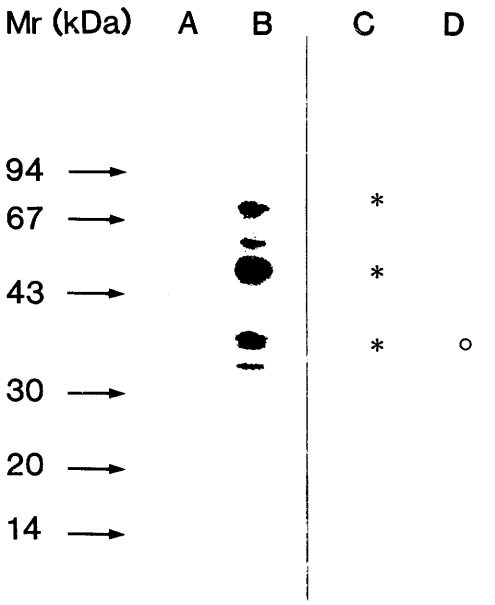


Figure 5 Binding of (^{3}H) AP_3-PL to ATP binding protein on the amyloplast envelope membrane. The amyloplast envelope was incubated with (^{3}H) AP_3-AL. Some amyloplast envelope was preincubated with 5 mM ATP. The ATP binding protein on the amyloplast envelope was determined by autoradiography, lance C and D. lane D, the amyloplast envelope was preincubated with unlabeled ATP. lane B, polypeptide pattern on the SDS-PAGE of amyloplast envelope incubated with (H^3) AP_3-PL and stained with CBB, lane A, the molecular weight marker.

Mg^{2+}-ATPase activity associated with amyloplast envelope was inhibited by ATP analog such as adenosine polyphosphopyridoxal (figure 4) and the inhibition could be protected by pretreatment with ATP (Harinasut *et al.*, 1988b). It has also been reported that this compound could bind with ATP-binding protein such as H^+-ATPase and adenylate kinase (Noumi *et al.*, 1987; Tagaya *et al.*, 1987). Therefore, the radioactivity of these compounds should be a useful tool for the identification of ATP-binding protein. We reported that among pyridoxal derivatives, adenosine triphosphopyridoxal was the most effective (Harinasut *et al.*, 1988b). The amyloplast envelope was incubated with (^{3}H) (AP_3-PL) and then visualized with the autoradiographic technique. Figure 5, lance C showed that amyloplast envelope contained at least three ATP-binding proteins which possessed 75, 54 and 37-kDa. Upon pretreatment with cold ATP, only the 37-kDa polypeptide bind with (^{3}H) (AP_3-PL) slightly better than the untreated sample, therefore these data support the assumption that the 37-kDa polypeptide may be an amyloplast envelope ATPase.

Conclusion : Triton X-100 could solubilize Mg^{2+}-ATPase of amyloplast envelope which may possess a 37-kDa subunit. Further experiment awaits to be carried out to verify the assumption by purification of the ATPase on DEAE-Sephadex chromatography as that previously reported on chloroplast envelope of pea (McCarty and Selman, 1986a).

ABBREVIATIONS

AP_3-PL	Adenosine triphosphopyridoxal.
CBB	Coomassie Brilliant Blue
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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