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Note

Enhanced solubilization of polygalacturonic acid synthase by ribonuclease treatment of cell homogenates

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Abstract The glycosyltransferases involved in plant cell wall biosynthesis are generally too low in concentration to allow for their purification. Therefore, the development of a method for enhanced solubilization of membrane proteins is necessary. Here, we report ribonuclease treatment of cell homogenates of azuki bean epicotyls. Polygalacturonic acid synthase, localized at the plant Golgi membrane, was enriched 2.0-fold in specific activity after ribonuclease treatment. Total enzyme activity per unit weight was also improved 2.1-fold. These data indicate that ribonuclease works to remove ribosomes from the microsomal fraction and enhances the solubilization of polygalacturonic acid synthase.

Key words: Enzyme, membrane protein, microsome, polygalacturonic acid synthase, ribonuclease.

Membrane proteins are generally difficult to biochemically characterize due to low intracellular concentrations and poor solubility in aqueous buffer. Even with the use of various detergents, it is sometimes not possible to obtain sufficient solubilized protein concentrations to allow for purification and/or biochemical characterization. Therefore, the development of more effective solubilization methods and enrichment of membrane proteins is greatly needed. Microsomes consisted of fragmented endoplasmic reticulum, endomembrane systems, and free ribosomes, are often prepared for biochemical analysis of membrane-localized proteins. To enrich membrane proteins of interest, microsomes are usually subfractioned using density gradient centrifugation. However, this method limits the volume of obtainable protein solution. When cell extracts are treated by ribonuclease (RNase), ribosomes would be expected to remove from the microsome fraction. This would have the effect of increasing the specific activity of membrane enzymes of interest in the solubilized microsome fraction. Here, we describe the investigation of the effect of RNase treatment of cell homogenate on the activity of membrane enzymes solubilized from microsome fractions.

As a model membrane enzyme, we choose polygalacturonic acid synthase (EC 2.4.1.43) (Schomburg et al. 2006) that catalyzes the synthesis of a backbone polysaccharide of pectic homogalacturonan and rhamnogalacturonan II. It catalyzes multiple transfers of galacturonic acid residues onto non-reducing end of oligogalacturonic acid to make polygalacturonic acid (Akita et al. 2002). This enzyme localized at the Golgi membrane (Sterling et al. 2001) has been fractioned in microsomes and solubilized with a buffer containing a detergent (Schomburg et al. 2006). However, its purification has not been achieved due to its low activity in solubilized enzyme solution. By way of comparison, solubilization of an animal Golgi-localized membrane enzyme, β-glucoside α1,3-xylosyltransferase, was also investigated to measure the effect of RNase treatment of cell homogenate.

The methods for solubilization of membrane proteins and for the assay of membrane enzymes are described here. Azuki bean epicotyls or Arabidopsis leaves (20 g of each) were pulverized in a mortar and pestle under liquid nitrogen. The powder was homogenized at 4°C for 20 min in buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-potassium hydroxide, pH 7.0, 25 mM potassium chloride, 50% (v/v) glycerol, 2 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin, and 1.0 mM phenylmethylsulfonyl fluoride). RNase A (protease-free, Calbiochem) at various concentrations was added to this buffer prior to homogenization. Magnesium chloride (finalconcentration 25 mM) was added to the supernatant (20,000×g, 10 min) followed by a 30 min incubation. This solution was centrifuged at 30,000×g for 45 min and the resulting pellet consisted of the microsomal fraction (Diesperger

Abbreviation: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RNase, ribonuclease.

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et al. 1974). This pellet was solubilized with a solubilization buffer (20 mM HEPES-potassium hydroxide, pH 7.0, 25 mM potassium chloride, 25% (v/v) glycerol, 2 mM EDTA, 100 mM ethylammonium nitrate, and 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) for 30 min at 4°C. The supernatant (30,000×*g*, 10 min) was used as the solubilized enzyme solution. Polygalacturonic acid synthase activity was measured as described previously (Akita et al. 2002) using fluorescence-labeled oligogalacturonic acid (Akita et al. 2002) and UDP-galacturonic acid (Ohashi et al. 2006). β-glucoside α1,3-xylosyltransferase activity, found in bovine liver microsomes, (Omichi et al. 1997) was also used in this study. Bovine liver (20 g) was homogenized in buffer (20 mM HEPES-sodium hydroxide, pH 7.4, 250 mM sucrose, 5 mM magnesium chloride) and incubated at 4°C for 20 min. RNase A at an appropriate concentration was added to this buffer just before homogenization. The supernatant (10,000×*g*, 25 min) was centrifuged at 105,000×*g*, 60 min and the resulting pellet consisted of the microsome fraction (Ishimizu et al. 2007). This pellet was solubilized with a buffer (20 mM HEPES-sodium hydroxide, pH 7.2, 20 mM magnesium chloride, 150 mM sodium chloride, 10% (v/v) glycerol, and 0.5% TritonX-100) for 30 min at 4°C. The supernatant (105,000×*g*, 60 min) was used as the enzyme source. β-glucoside α1,3-xylosyltransferase activity was measured as described previously (Ishimizu et al. 2007) using fluorescence-labeled glucoside (Ishimizu et al. 2007) and UDP-xylose (Ishimizu et al. 2005).

Incubation of cell homogenate from azuki bean epicotyls with RNase A at 4°C for 20 min increased the specific activity of polygalacturonic acid synthase in the solubilized enzyme solution as expected (Table 1). An increase was observed at a concentration of RNase A as low as 0.5 $\mu g\,ml^{-1}$. In the presence of 50 $\mu g\,ml^{-1}$ RNase A, a two-fold increase in specific activity of the solubilized enzyme solution was observed. Total activity was also improved 2.1-fold. This means that the solubilization of proteins from microsomes was increased. Such an increase in specific activity and total activity of the enzyme in the solubilized microsome fraction is advantageous for biochemical characterization of membrane proteins.

The effect of RNase A on enzyme activity in the solubilized enzyme solution was also investigated with other starting materials. Polygalacturonic acid synthase from Arabidopsis leaves and β-glucoside α1,3-xylosyltransferase from bovine livers were examined. The solubilization of the enzyme from Arabidopsis leaves also benefited from RNase treatment, although the effect was less than that from azuki bean epicotyl enzymes (Table 2). However, RNase treatment of bovine liver homogenate did not cause any increase in α1,3-xylosyltransferase activity (Table 2). Furthermore, increasing the concentration of RNase A (up to 5 $\mu g\,ml^{-1}$) did not have any effect (data not shown). The differences in the effect of RNase A treatment observed with the different starting materials seem to be due to varying ribosome contents in the various materials. The ribosome content in bacteria, yeast, and some variations of plant cells is known to be proportional to their respective growth rates (Bremer and Dennis 1996; Ingle and Hageman 1964; Waldron and Lacroute 1975). Azuki bean epicotyl is a plant material with high growth velocity (7 cm day^{-1}) (Yasui et al. 2009) and is likely to contain many ribosomes per cell. In contrast, hepatocytes contain smooth endoplasmic reticulum without ribosomes. Thus, the ribosome content in bovine liver seems to be relatively low. This method appears to be useful for the preparation of solubilized membrane proteins from growing cells in which the ribosome concentration is relatively high.

RNase digestion of microsome fractions has previously been attempted (Novikoff et al. 1954; Yamamoto and Furuya 1979). In these attempts, RNase

Table 1 Concentration dependence of RNase A to cell homogenates and polygalacturonic acid synthase activity from azuki bean epicotyls

Concentration of RNase A ($\mu g\,ml^{-1}$)	Polygalacturonic acid synthase activity ± SD	
	Relative specific activity	Relative total activity
None	1.0	1.0
0.5	1.3 ± 0.2	1.5 ± 0.1
1	1.5 ± 0.2	1.7 ± 0.2
5	1.4 ± 0.2	1.6 ± 0.1
10	1.5 ± 0.2	1.6 ± 0.2
50	2.0 ± 0.4	2.1 ± 0.2
100	1.3 ± 0.1	1.7 ± 0.2
500	1.7 ± 0.2	1.9 ± 0.3

Activities are expressed relative to those from solubilized enzyme solutions prepared without RNase treatment. These values are the averages of three independent experiments. SD represents standard deviation.

Table 2 Effects of RNase A (50 $\mu g\,ml^{-1}$) on yields of membrane protein extraction from different starting materials

Materials (membrane enzymes characterized)	Enzyme activity ± SD	
	Relative specific activity	Relative total activity
Azuki bean epicotyls (Polygalacturonic acid synthase)	2.0 ± 0.4	2.1 ± 0.2
Arabidopsis leaves (Polygalacturonic acid synthase)	1.5 ± 0.2	1.6 ± 0.5
Bovine liver (β-glucoside α1,3-xylosyltransferase)	1.0 ± 0.1	1.1 ± 0.1

Activities are expressed relative to those from solubilized solutions prepared without RNase treatment. The values are averages of three independent experiments. SD represents standard deviation.

digestion was conducted at 37°C, and as a result, almost all enzymes were denatured (Novikoff et al. 1954). In this study, crude extracts including the microsome fraction were incubated with RNase A at 4°C. Due to this, enzymes were not inactivated, and in fact, the total enzymatic activity was increased. The levels of both relative total activity and specific activity of the enzyme increased by RNase treatment (Table 1), indicating that the solubility of proteins in the microsomes recovered after RNase treatment was increased.

A number of membrane proteins remain unidentified even in organisms with characterized genomes. This study is a useful addition to methods for the preparation of membrane enzymes in solubilized microsome fractions for biochemical characterization and/or purification. In addition, we have developed another method for enhancing solubilization of membrane proteins from microsomes using alkylamines or polyamines (Yasui et al. 2010). This method enhanced solubilization of polygalacturonic acid synthase up to 9.9-fold using optimum conditions. A combination of these two methods appears to provide insight into the purification of low abundant membrane proteins including glycosyltransferases involved in plant cell wall biosynthesis.

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