Enhanced solubilization of membrane proteins by alkylamines and polyamines

Kazutoshi Yasui,¹ Masamichi Uegaki,¹ Kentaro Shiraki,² and Takeshi Ishimizu^{1*}

¹Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

²Institute of Applied Physics, University of Tsukuba, Tsukuba, Ibaraki 305-8573, Japan

Received 22 July 2009; Revised 18 December 2009; Accepted 20 December 2009

DOI: 10.1002/pro.326

Published online 6 January 2010 proteinscience.org

Abstract: Around 25% of proteins in living organisms are membrane proteins that perform many critical functions such as synthesis of biomolecules and signal transduction. Membrane proteins are extracted from the lipid bilayer and solubilized with a detergent for biochemical characterization; however, their solubilization is an empirical technique and sometimes insufficient quantities of proteins are solubilized in aqueous buffer to allow characterization. We found that addition of alkylamines and polyamines to solubilization buffer containing a detergent enhanced solubilization of membrane proteins from microsomes. The solubilization of polygalacturonic acid synthase localized at the plant Golgi membrane was enhanced by up to 9.9-fold upon addition of spermidine to the solubilization buffer. These additives also enhanced the solubilization of other plant membrane proteins localized in other organelles such as the endoplasmic reticulum and plasma membrane as well as that of an animal Golgi-localized membrane protein. Thus, addition of alkylamines and polyamines to solubilization buffer is a generally applicable method for effective solubilization of membrane proteins. The mechanism of the enhancement of solubilization is discussed.

Keywords: alkylamine; membrane protein; polyamine; polygalacturonic acid synthase; solubilization

Introduction

Membrane proteins function in critical biological processes such as synthesis of biomolecules, energy metabolism, the transduction of signals, and the transport of solutes across membranes. Around 25% of naturally occurring proteins are predicted to be membrane proteins^{1,2}; however, they have been less studied than soluble proteins mainly because of low abundance in cells and their poor recovery in solution. In addition, it is difficult to produce recombinant membrane proteins in heterologous cells. Furthermore, there are no standardized methods for

preparation of solubilized membrane proteins and for purification of membrane proteins from living cells. These experimental conditions must be optimized for every membrane protein, and it often takes a great deal of time and effort. Therefore, more effective, standardized methods of handling membrane proteins are still actively sought.

The procedure for the purification of a membrane protein begins with its solubilization. Membrane proteins have poor recovery in aqueous buffer due to their being embedded in a lipid bilayer and due to their hydrophobic nature. Solubilization of membrane proteins has been achieved in appropriate buffers with mild detergents. Numerous detergents for solubilization of membrane proteins have been investigated.^{3,4} This solubilizing step is an empirical technique and the selection of detergent and optimization of solubilization conditions are determined by trial and error experiments that depend on the protein of interest. Despite laborious experiments, sufficient levels of solubilization of a membrane protein are sometimes not accomplished.

Grant sponsor: Grants-in-Aid for Special Research on Priority Areas; Grant number: 21024006; Grant sponsors: Grant-in-Aid for Scientific Research for Plant Graduate Students from Nara Institute of Science and Technology. Both grants are from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

*Correspondence to: Takeshi Ishimizu, Graduate School of Science, Osaka University, 1-1 Machikaneyamacho, Toyonaka, Osaka 560-0043, Japan. E-mail: txi@chem.sci.osaka-u.ac.jp

Polygalacturonic acid synthase localized at the plant Golgi membrane⁵ is one such example. Although its purification has been attempted since detection of its activity from plant sources in the 1960s,⁶ this has not been achieved because the enzyme activity in the solubilized solution is too low.

The effects of various additives on the solubilization of membrane proteins have been investigated.⁷ Polyols such as glycerol, polyethylene glycol, and sucrose enhance the stability of membrane proteins in solution presumably by preventing unfavorable hydrophobic interactions. The addition of sufficient EDTA and EGTA is useful for inhibiting proteolysis to stabilize membrane proteins in solution. Protease inhibitors such as phenylmethylsulfonyl fluoride, leupeptin, aprotinin, E-64, and pepstatin are also added to buffers to increase stabilization. Additives for enhancing solubilization of membrane proteins can give a significant advantage in the purification of membrane proteins. Nondetergent sulphobetaines were reported to enhance the recovery of some kinds of membrane proteins.⁸ Other than those listed above, no other additives have been reported to be generally effective for enhancing solubilization of membrane proteins.

In this study, we investigated alkylamines or polyamines as additives for enhancing solubilization of membrane proteins. Polyamines such as spermine and spermidine, which are abundantly synthesized in the cells of all living organisms, are involved in a number of processes, but their specific molecular mechanism is largely unknown.^{9,10} Interestingly, polyamines are important modulators of some membrane proteins including a variety of channels and receptors.¹⁰ In addition, they have been shown to prevent thermal inactivation and aggregation of protein.^{11,12} Ethylammonium nitrate, an ionic liquid, is also known to prevent aggregation of denatured proteins.¹³ This solvent has also been used as a protein crystallization reagent.¹⁴ The moderate hydrophobicity and cationic character of alkylamines and polyamines are expected to assist in the solubilization of membrane proteins. In addition, the positive charge of these molecules has the potential to interact with the negatively charged lipid bilayers and is thus expected to further help solubilization of membrane proteins. Here, we demonstrate that the addition of alkylamines and polyamines to a solubilization buffer containing a detergent enhances the solubilization of membrane proteins.

Results

Enhanced solubilization of polygalacturonic acid synthase by alkylamines and polyamines

Polygalacturonic acid synthase localized at the plant Golgi membrane has previously been solubilized from a microsome fraction with a solubilization buffer containing CHAPS.^{15–17} Even when a deter-

Figure 1. Structure of alkylamines and polyamines used in this study.

gent was used for its solubilization, insufficient amounts of the enzyme were obtained to allow purification. Thus, more effective solubilization conditions are required. We investigated the effects of three kinds of alkylamines (ethylammonium chloride, ethylammonium nitrate, and propylammonium chloride) and three polyamines (putrescine, spermidine, and spermine) (the structures of which are shown in Fig. 1) on solubilization of this enzyme. We prepared microsomal fractions from azuki bean epicotyls. When the enzyme was solubilized from the microsomal fraction with a solubilization buffer (containing 20 mM CHAPS) supplemented with each additive at a concentration of 100 mM, enhanced solubilization of the enzyme was observed in all cases. The specific activity of the enzyme increased from 2.2- to 8.2-fold, while the total activity also increased from 2.3- to 6.3-fold (Table I). Of the additives, spermidine was the most effective in the solubilization of the enzyme. A notable difference in the effect of the counter anion (ethylammonium chloride and ethylammonium nitrate) was not observed. Such enhanced solubilization was also observed when Triton X-100 or sodium cholate was used as the detergent instead of CHAPS. In the presence of 0.5% Triton X-100, ethylammonium nitrate (100 mM) and spermidine (100 mM) enhanced the solubilization of polygalacturonic acid synthase by 1.8- and 1.6-fold, respectively. In the presence of 1% sodium cholate, both two additives enhanced the solubility of this membrane enzyme by 1.6-fold.

Dependence of solubilization of polygalacturonic acid synthase on alkylamine and polyamine concentration

Once we had shown that various alkylamines and polyamines enhance the solubilization of a membrane enzyme, the relationship between enhancement of the total activity of the enzyme solubilized and concentration of ethylammonium nitrate and spermidine was investigated. Enhancement was observed at a concentration of ethylammonium nitrate as low as 10 mM [Fig. 2(A)]. Under optimum conditions (100 mM), a

Table I. Effects of Alkylamines and Polyamines on Solubilization of Polygalacturonic Acid Synthase

Additive	Polygalacturonic acid synthase activity	
	Specific activity (mU/mg protein)	Total activity (mU/g fresh weight)
No additive	0.17 (1.0)	0.032 (1.0)
Ethylammonium chloride	0.54 (3.2)	0.14 (4.4)
Ethylammonium nitrate	0.37 (2.2)	0.11 (3.4)
Propylammonium chloride	0.36 (2.2)	0.11 (3.4)
Putrescine	0.41 (2.4)	0.12 (3.8)
Spermidine	1.4 (8.2)	0.20 (6.3)
Spermine	0.94 (5.5)	0.074 (2.3)

Each additive was added to solubilization buffer at a concentration of 100 mM. The parenthetic values indicate activities relative to those of the enzyme solution prepared without additive.

3.4-fold increase of total activity was observed. However, as the concentration increased, total activity of the enzyme solubilized gradually decreased. The relationship between solubilization and concentration for spermidine was similar to that for ethylammonium nitrate [Fig. 2(B)]. Thus, enhancement of solubilization was observed even at a concentration of 10 mM. Maximum enhancement (9.9-fold) was observed at 50 mM spermidine. As was the case with ethylammonium nitrate, as the spermidine concentration was increased, the amount of enzyme solubilized decreased drastically.

Enhanced solubilization of other membrane enzymes by alkylamines and polyamines

The alkylamines and polyamines used in this study were shown to enhance the solubilization of polygalacturonic acid synthase from microsome fractions. Subsequently, we investigated whether these reagents enhance the solubilization of other membrane enzymes. Polygalacturonic acid synthase is localized at the Golgi membrane. We therefore chose three other plant enzymes localized at membranes other than the Golgi. The solubilization of NADH-dependent cytochrome c reductase localized at the endoplasmic reticulum was 2.2- to 2.5-fold enhanced by addition of 100 mM ethylammonium nitrate or 50 mM spermidine (Table II). Enhancement of solubili-

zation of mitochondrial localized cytochrome c oxidase was not observed to the same extent (1.1-fold) because most of the mitochondrial fragments were not contained in the microsome fractions. The solubilization of γ -glutamyl transpeptidase localized at the plasma membrane was enhanced 3.8- and 3.4-fold by ethylammonium nitrate and spermidine, respectively. Table II summarizes how the addition of alkylamines and polyamines to solubilization buffer is applicable for effective solubilization of these plant membrane proteins. The values of relative specific activity are not much different from those of total activity, indicating that the solubilization of microsomal proteins other than the membrane enzymes tested was also enhanced by ethylammonium nitrate and spermidine.

The effects of these additives were also investigated for an animal membrane protein, namely bovine liver β -glucoside α 1,3-xylosyltransferase.¹⁸ The relationship between solubilization of this enzyme from a bovine liver microsomal fraction and the concentration of the additives is shown in Figure 3. In the absence of additives the enzyme activity was 1.37 U/g fresh weight. The solubilization of this enzyme was enhanced by ethylammonium nitrate and spermidine up to 7.1- and 4.7-fold, respectively. The optimum concentrations of the additives (500 mM ethylammonium nitrate and 100–500 mM spermidine) are

Figure 2. Relationship between solubilization of polygalacturonic acid synthase and concentration of ethylammonium nitrate and spermidine in solubilization buffer. (A) Ethylammonium nitrate and (B) spermidine.

Table II. Effects of Ethylammonium Nitrate and Spermidine on Solubilization of Various Plant Membrane Enzymes

Enzyme	Specific activity		Ethylammonium nitrate	Spermidine
	Total activity	No additive		
Polygalacturonic acid synthase (Golgi)		1.0	1.5	6.8
		1.0	2.8	9.9
NADH-dependent cytochrome c reductase (endoplasmic reticulum)		1.0	1.9	2.8
		1.0	2.2	2.5
		1.0	2.2	2.5
Cytochrome c oxidase (mitochondria)		1.0	1.1	1.5
		1.0	1.1	1.1
γ -Glutamyl transpeptidase (plasma membrane)		1.0	3.7	4.4
		1.0	3.8	3.4

Ethylammonium nitrate (100 mM) or spermidine (50 mM) was added to solubilization buffer. Upper and lower values in a column represent specific activity and total activity of the enzymes, respectively. The values indicate activities relative to those of the enzyme solution prepared without additive.

around fivefold higher than that for plant membrane proteins. The higher concentration of additives decreased the recovered yield of the enzyme.

Alkylamines and polyamines do not stabilize polygalacturonic acid synthase

How do alkylamines and polyamines enhance the solubilization of membrane proteins? We investigated possible explanations by analyzing the solubilization of polygalacturonic acid synthase as a model membrane protein. First, we investigated the contribution of these additives for stabilization of the protein in aqueous buffer. The membrane enzymes solubilized with various concentrations of additives were incubated at 4°C for 24 h. Subsequent enzyme activities were compared with that of the enzyme solubilized without additives. The results showed that the additives did not stabilize polygalacturonic acid synthase but rather destabilized it [Fig. 4(A)]. Thus, the greater the concentration, the lower the enzyme activity. This indicates that ethylammonium nitrate is a weak protein denaturant. Spermidine also destabilized the enzyme solubilized in aqueous buffer [Fig. 4(B)]. The denaturing effect of spermidine was a lit-

tle stronger than that of ethylammonium nitrate. These results argue against the idea that the enhanced solubilization of membrane proteins by alkylamines and polyamines is due to stabilization of the membrane proteins.

Alkylamines and polyamines do not activate polygalacturonic acid synthase

Subsequently, it was investigated whether the additives contribute to the activation of the enzyme. Ethylammonium nitrate or spermidine (100 mM) was added to the enzyme solution prepared without additives during the assay. Neither reagent activated polygalacturonic acid synthase but in fact rather inhibited it (Table III). This indicates that the increased values of enzyme activity in Tables I and II obtained upon addition of alkylamines and polyamines are not due to the activation of the enzyme by these reagents.

Alkylamines and polyamines do not act as alternatives to detergent

We checked whether alkylamines and polyamines could act as alternatives to detergents and thereby enhance the solubilization of membrane proteins.

Figure 3. Relationship between solubilization of β -glucoside α 1,3-xylosyltransferase and concentration of ethylammonium nitrate and spermidine in solubilization buffer. (A) Ethylammonium nitrate and (B) spermidine.

Figure 4. Stability of polygalacturonic acid synthase solubilized with a buffer containing (A) ethylammonium nitrate or (B) spermidine at various concentrations. Enzyme activity was measured after incubation of the solubilized enzyme at 4°C for 24 h. Remaining activities relative to the enzyme prepared without additives are plotted.

When the proteins were solubilized from the microsome fraction, 100 mM ethylammonium nitrate or 100 mM spermidine was added to the solubilization buffer instead of 20 mM CHAPS. These reagents worked for solubilization of polygalacturonic acid synthase to a small extent (Table IV) but this does not explain the reason for enhanced solubilization of membrane proteins by these reagents.

Discussion

Alkylamines and polyamines were found to be effective additives for solubilization of polygalacturonic acid synthase from microsome fractions. Of the additives, spermidine was the most effective for plant membrane proteins (Table I). With polygalacturonic acid synthase, which has not previously been purified, addition of 50 mM spermidine to solubilization buffer enhanced the solubilization by 9.9-fold (Fig. 2). Because one of the reasons for the lack of purification of the enzyme is low enzyme activity in solubilized microsomal fractions, this method opens the way for its purification. These additives also enhanced the solubilization of other plant membrane enzymes (Table II) and an animal membrane enzyme (Fig. 3). This implies that these additives are effective for solubilization of membrane proteins from various sources. This solubilization method may also be applicable to the biochemical characterization of especially low abundance membrane proteins such as glycosyltransferases.

Although the present report does not provide a precise mechanism to explain the enhanced solubilization of membrane proteins by alkylamines and polyamines, we would like to discuss possible mechanisms. Based on the data described here, the higher enzyme activity of the enzyme solubilized with the additives was shown to not be caused by the additives working as stabilizers (Fig. 4) or activators (Table III) of the enzymes. Indeed, it has been reported that spermidine slightly denatures proteins.^{11,19}

Thus, upon purification of a membrane protein solubilized by this method, it will be necessary to remove the additive from the protein solution as soon as possible. When these reagents were used instead of a detergent (CHAPS), some solubilization of a membrane protein was observed (Table IV). But this observation does not explain the size of the enhancement of the solubilization of membrane proteins by these reagents.

This enhanced solubilization of membrane proteins was not specific to those solubilized with CHAPS. Alkylamines and polyamines in the presence of detergents other than CHAPS also enhanced solubilization of membrane enzymes. Thus, enhanced solubilization of bovine xylosyltransferase upon addition of additives was also observed with the use of Triton X-100 (Fig. 3). When Triton X-100 or sodium cholate was used as a detergent for solubilization of polygalacturonic acid synthase, enhanced solubilization upon addition of additives was also observed.

The reagents used in this study have a number of common structural features as they have relatively simple structures that are composed of multivalent amines. Because of their cationic nature, alkylamines and polyamines possibly bind to anionic sites of organic compounds. In fact, the polyamines have been reported to interact with microsomes or a lipid bilayer membrane and cause their

Table III. Activating Effects of Ethylammonium Nitrate or Spermidine on Polygalacturonic Acid Synthase Activity

Additive	Enzyme activity (μU/mL)
No additive	372 (1.00)
Ethylammonium nitrate	43.2 (0.12)
Spermidine	9.4 (0.03)

Each additive was added to reaction buffer at a concentration of 100 mM. The parenthetical values indicate activities relative to those of the enzyme solution without additive.

Table IV. Solubilization Effects of Ethylammonium Nitrate or Spermidine Acting as Detergents

Additive	Polygalacturonic acid synthase activity	
	Specific activity (mU/mg protein)	Total activity (μ U/g fresh weight)
CHAPS (20 mM)	0.28 (1.0)	69 (1.0)
Ethylammonium nitrate (100 mM)	0.11 (0.4)	21 (0.3)
Spermidine (100 mM)	0.15 (0.5)	22 (0.3)

Each additive (100 mM) was added to solubilization buffer instead of detergent. The parenthetical values indicate activities relative to those of the enzyme solution prepared in the presence of 20 mM CHAPS.

destabilization.^{20–22} Furthermore, they were observed to bind to microsomes but not to microsomal proteins.²⁰ These results suggest the possibility that these reagents interact with phospholipids and enhance delipidation of membrane proteins. Thus, they may assist in the release of membrane proteins from lipid bilayers, so that solubilization of membrane proteins is enhanced.

When a membrane enzyme from bovine liver was solubilized from microsomes, around a fivefold higher concentration of ethylammonium nitrate and spermidine was needed for optimum solubilization compared to that used for microsomal proteins of azuki bean epicotyls. The compositions of phospholipids in lipid bilayers of plants and animals are totally different.^{23–25} Plant microsomes have a higher content of phosphatidylethanolamine (PE) and phosphatidylinositol (PI) and a lower content of phosphatidylcholine (PC) than animal liver microsomes. The binding strength of polyamines to phospholipids has been reported to be in the order $PI > PE > PC$.²⁰ This probably explains the difference in the observed optimum concentration of additives for membrane protein solubilization from animals and plants. Thus, higher concentrations of polyamines may be required for effective solubilization of membrane proteins of animal liver microsomes in which the PC content is higher than in plant microsomes. Furthermore, binding of spermidine to rat liver microsomes is reported to be stronger than that of spermine.²⁶ This report is consistent with our data, which demonstrate that spermidine is a more effective reagent for enhancing the solubilization of microsomal proteins (Table I). The phospholipid compositions of lipid bilayers are not constant between organisms. However, the addition of alkylamines and polyamines for enhancing solubilization of membrane proteins appears to be applicable to various organisms, although the optimum reagent and concentration may need to be determined for each sample.

It has been frequently reported that membrane enzymes are activated by polyamines.^{27–29} In most reports, enzyme activities were measured using microsome fractions. Based on this study, polyamines may not activate membrane enzymes but in most cases enhance their solubilization from microsomes.

This study shows that alkylamines and polyamines are effective additives for the solubilization of some plant membrane proteins and animal Golgi-localized proteins. Further application of this method to membrane proteins from other organisms may allow this method to be generally applied for the effective solubilization of membrane proteins.

Materials and Methods

Materials

Ethylammonium nitrate was prepared by adding equimolar amounts of ethylamine (70%) to nitric acid (61%) drop-by-drop with stirring and cooling in an ice bath.³⁰ The resulting salt solution was concentrated *in vacuo* on a rotary evaporator. The concentrated solution was dried by lyophilization for 2 days. The density (1.20 g cm^{-3}) was close to the literature value.^{31,32}

Ethylammonium chloride, propylammonium chloride, and putrescine dihydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Spermidine trihydrochloride and spermine tetrahydrochloride were from MP Biomedicals (Irvine, CA). UDP-galacturonic acid was enzymatically synthesized as reported previously.³³ UDP-xylose was chemically synthesized as described previously.³⁴ Fluorescent-labeled oligogalacturonic acids were prepared as described previously.^{16,35} 2-[(2-Pyridyl)amino]ethyl β -glucoside was prepared by transglycosylation activity of β -glucosidase as described.¹⁸ All other chemicals used were of the highest grade commercially available.

Preparation of the solubilized membrane enzyme

Azuki beans (*Vigna angularis*) were germinated and grown at an ambient temperature in the dark for around 10 days. Their epicotyls (50 g) were pulverized with a mortar and a pestle under liquid nitrogen. The ground powder was stirred at 4°C for 20 min with 50 mL of a homogenizing buffer (50 mM HEPES-KOH, pH 7.0, 25 mM KCl, 50% (v/v) glycerol, 2 μ g/mL aprotinin, 5 μ g/mL leupeptin, 0.7 μ g/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride). To the supernatant (20,000g, 10 min), MgCl_2 (final 25 mM) was added and incubated at 4°C for

30 min. This solution was centrifuged at 30,000g for 30 min to obtain the microsomal fraction as pellets.³⁶ This fraction was solubilized with 1 mL of a solubilization buffer (20 mM HEPES-KOH, pH 7.0, 25 mM KCl, 25% (v/v) glycerol, 2 mM EDTA, 20 mM CHAPS) at 4°C for 20 min. The additives (alkylamines or polyamines) were added to a solubilization buffer before solubilization. The supernatant (30,000g, 10 min) was used as a solubilized enzyme solution.

Bovine liver (20 g) was homogenized with 180 mL of 20 mM HEPES-NaOH buffer, pH 7.4, containing 250 mM sucrose and 5 mM MgCl₂. The homogenate was centrifuged at 10,000g at 4°C for 25 min. The supernatant was further centrifuged at 105,000g at 4°C for 1 h. The pellets were collected and the protein was solubilized with 30 mL of a solubilization buffer (20 mM HEPES-NaOH buffer, pH 7.2, containing 20 mM MgCl₂, 150 mM NaCl, 10% (v/v) glycerol, and 0.5% Triton X-100). The additives (alkylamines or polyamines) were added to a solubilization buffer before solubilization. The supernatant obtained after centrifugation (105,000g for 1 h) was used as a solubilized enzyme solution.¹⁸

Enzyme assay

Enzyme activities were measured just after solubilization of membrane proteins from microsomal fractions. Polygalacturonic acid synthase activity was measured using pyridylaminated oligogalacturonic acids and UDP-galacturonic acid as substrates.^{16,35} A reaction mixture containing 0.5 mM UDP-galacturonic acid, 5 μ M pyridylaminated oligogalacturonic acid with degree of polymerization of 12, 33 mM HEPES-NaOH, pH 7.0, 17 mM KCl, 5 mM MnCl₂, 0.13M sucrose, 0.033% bovine serum albumin, 10 mM CHAPS, and the enzyme solution was incubated at 30°C for 10 min. Transferred galacturonic acid was quantified from the chromatogram peak area obtained from anion-exchange chromatography with fluorescence detection. One unit of enzyme activity was defined as the amount of the enzyme that transferred 1 μ mol of galacturonic acid onto oligogalacturonic acid per min under the conditions described earlier.

NADH-dependent cytochrome c reductase activity was assayed at an ambient temperature for 5 min in a reaction mixture containing 20 mM potassium phosphate buffer, pH 7.2, 0.2 mM NADH, 0.02 mM bovine heart cytochrome c, 10 mM potassium cyanide, 1 μ M antimycin A, and the enzyme solution.³⁷ The amount of cytochrome c reduced was estimated by increase in absorbance at 550 nm using an extinction coefficient for cytochrome c of 18.5 mM⁻¹ cm⁻¹.

Cytochrome c oxidase activity was measured at an ambient temperature for 15 min in a reaction mixture containing 45 mM potassium phosphate

buffer, pH 7.2, 0.01% digitonin, 0.02 mM reduced bovine heart cytochrome c, and the enzyme solution.³⁸ The reduced cytochrome c was prepared by chemical reduction with 20 mM sodium dithionite on ice for 10 min. The enzyme activity was quantified by reduction of absorbance at 550 nm.

γ -Glutamyl transpeptidase activity was performed with γ -glutamyl-*p*-nitroanilide (GPNA) as a donor substrate and glycylglycine as an acceptor substrate.³⁹ The reaction was carried out in a mixture of 1 mM GPNA, 40 mM glycylglycine, 0.1M Tris-HCl, pH 7.5, and the enzyme solution at 30°C for 1 h. Liberated *p*-nitroaniline was quantified at 405 nm.

β -Glucoside α 1,3-xylosyltransferase activity¹⁸ in the solubilized enzyme solution prepared from bovine liver was assayed using 2-[(2-pyridyl)amino]ethyl β -glucoside¹⁸ as an acceptor substrate and UDP-xylose³⁴ as a donor substrate. The reaction mixture (20 mM HEPES-NaOH, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 20 mM MnCl₂, 1 mM 2-[(2-pyridyl)amino]ethyl β -glucoside, and 1.3 mM UDP-xylose) was incubated at 37°C for 12 h. The substrate and product were analyzed on a Cosmosil 5C18-P reversed-phase column (4.6 mm $\times$ 150 mm, Nacalai Tesque) with isocratic elution in 50 mM ammonium acetate buffer, pH 4.5, at a flow rate of 2 mL/min. They were quantified by their fluorescence (excitation wavelength, 320 nm; emission wavelength, 400 nm).

We performed at least two independent experiments for each table and figure. The values of relative enzyme activities in tables and figures are typical data from a single experiment. The values are reproducible values, although the actual enzyme activity differed between each experiment because of the use of different biological sources.

References

1. Wallin E, von Heijne G (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci* 7: 1029–1038.
2. Stevens TJ, Arkin IT (2000) Do more complex organisms have a greater proportion of membrane proteins in their genomes? *Proteins* 39:417–420.
3. le Maire M, Champeil P, Møller JV (2000) Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim Biophys Acta* 1508:86–111.
4. Arnold T, Linke D, The use of detergents to purify membrane proteins. In: Coligan JE, Dunn BM, Speicher DW, Wingfield PT, Eds. (2008) *Current protocols in protein science*, Vol. 53. Hoboken: Wiley, pp 4.8.1–4.8.30.
5. Schomburg D, Schomburg I, Chang A, Polygalacturonate 4- α -galacturonosyltransferase. In: (2006) *Springer handbook of enzymes*, Vol. 31. Heidelberg: Springer, pp 407–411.
6. Villemez CL, Lin T-Y, Hassid WZ (1965) Biosynthesis of the polygalacturonic acid chain of pectin by a

- particulate enzyme preparation from *Phaseolus aureus* seedlings. *Proc Natl Acad Sci USA* 54:1626–1632.
7. Hjelmeland LM (1990) Solubilization of native membrane proteins. *Methods Enzymol* 182:253–264.
 8. Blisnick T, Morales-Betoulle ME, Vuillard L, Rabilloud T, Breton CB (1998) Non-detergent sulphobetaines enhance the recovery of membrane and/or cytoskeleton-associated proteins and active proteases from erythrocytes infected by *Plasmodium falciparum*. *Eur J Biochem* 252:537–541.
 9. Wortham BW, Patel CN, Oliveira MA (2007) Polyamines in bacteria: pleiotropic effects yet specific mechanisms. *Adv Exp Med Biol* 603:106–115.
 10. Kusano T, Berberich T, Tateda C, Takahashi Y (2008) Polyamines: essential factors for growth and survival. *Planta* 228:367–381.
 11. Kudou M, Shiraki K, Fujiwara S, Imanaka T, Takagi M (2003) Prevention of thermal inactivation and aggregation of lysozyme by polyamines. *Eur J Biochem* 270:4547–4554.
 12. Hamada H, Takahashi R, Noguchi T, Shiraki K (2008) Differences in the effects of solution additives on heat- and refolding-induced aggregation. *Biotechnol Prog* 24:436–443.
 13. Summers CA, Flowers RA, II (2000) Protein renaturation by the liquid organic salt ethylammonium nitrate. *Protein Sci* 9:2001–2008.
 14. Garlitz JA, Summers CA, Flowers RA, II, Borgstahl GEO (1999) Ethylammonium nitrate: a protein crystallization reagent. *Acta Cryst D* 55:2037–2038.
 15. Doong RL, Mohnen D (1998) Solubilization and characterization of a galacturonosyltransferase that synthesizes the pectic polysaccharide homogalacturonan. *Plant J* 13:363–374.
 16. Ohashi T, Ishimizu T, Akita K, Hase S (2007) Stabilization and minimum active component of polygalacturonic acid synthase involved in pectin biosynthesis. *Biosci Biotechnol Biochem* 71:2291–2299.
 17. Yasui K, Jinno J, Ohashi T, Ishimizu T, In vitro synthesis of polygalacturonic acid. In: Voragen F, Schols H, Visser R, Eds. (2009) *Pectins and pectinases*. Dordrecht, The Netherlands: Kluwer, pp 167–175.
 18. Ishimizu T, Sano K, Uchida T, Teshima H, Omichi K, Hojo H, Nakahara Y, Hase S (2007) Purification and substrate specificity of UDP-D-xylose:β-D-glucoside α-1,3-D-xylosyltransferase involved in the biosynthesis of the Xylα1-3Xylα1-3Glcβ1-O-Ser on epidermal growth factor-like domains. *J Biochem* 141:593–600.
 19. Hamada H, Arakawa T, Shiraki K (2009) Effect of additives on protein aggregation. *Curr Pharm Biotechnol* 10:400–407.
 20. Igarashi K, Sakamoto I, Goto N, Kashiwagi K, Honma R, Hirose S (1982) Interaction between polyamines and nucleic acids or phospholipids. *Arch Biochem Biophys* 219:438–443.
 21. Zheliaskova A, Naydenova S, Retrov AG (2000) Interaction of phospholipids bilayers with polyamines of different length. *Eur Biophys J* 29:153–157.
 22. Spassova M, Mellor R, Petrov AG, Usherwood PNR (1998) Philantotoxin-343 and spermine form ion pores in lipid bilayers. *CR Acad Bulg Sci* 51:41–44.
 23. Abdelkader AB, Mazliak P (1970) Lipid exchange between mitochondria, microsomes and cytoplasmic supernatant of cells of potatoes and cauliflower. *Eur J Biochem* 15:250–262.
 24. Getz GS, Bartley W, Lurie D, Notton BM (1968) The phospholipids of various sheep organs, rat liver and of their subcellular fractions. *Biochim Biophys Acta* 152:325–339.
 25. Bergelson LD, Dyatlovitskaya EV, Torkhovskaya TI, Sorokina IB, Gorkova NP (1970) Phospholipid composition of membranes in the tumor cell. *Biochim Biophys Acta* 210:287–298.
 26. Watanabe S, Kusama-Eguchi K, Kobayashi H, Igarashi K (1991) Estimation of polyamine binding to macromolecules and ATP in bovine lymphocytes and rat liver. *J Biol Chem* 266:20803–20809.
 27. Schubert F (1989) Influence of polyamines on membrane functions. *Biochem J* 260:1–10.
 28. Navaratnam N, Virk SS, Ward S, Kuhn NJ (1986) Cationic activation of galactosyltransferase from rat mammary Golgi membranes by polyamines and by basic peptides and proteins. *Biochem J* 239:423–433.
 29. Kauss H, Jeblick W (1986) Synergistic activation of 1,3-β-D-glucan synthase by Ca²⁺ and polyamines. *Plant Sci* 43:103–107.
 30. Evans DF, Yamauchi A, Roman R, Casassa EZ (1982) Micelle formation in ethylammonium nitrate, a low-melting fused salt. *J Colloid Interface Sci* 88:89–96.
 31. Sugden S, Wilkins H (1929) The parachor and chemical constitution. XII. Fused metals and salts. *J Chem Soc* 1291–1298.
 32. Allen M, Evans DF, Lumry R (1985) Thermodynamic properties of the ethylammonium nitrate + water system: partial molar volumes, heat capacities, and expansivities. *J Sol Chem* 14:549–560.
 33. Ohashi T, Cramer N, Ishimizu T, Hase S (2006) Preparation of UDP-galacturonic acid using UDP-sugar pyrophosphorylase. *Anal Biochem* 352:182–187.
 34. Ishimizu T, Uchida T, Sano K, Hase S (2005) Chemical synthesis of uridine 5'-diphospho-α-D-xylopyranose. *Tetrahedron: Asymmetry* 16:309–311.
 35. Akita K, Ishimizu T, Tsukamoto T, Ando T, Hase S (2002) Successive glycosyltransfer activity and enzymatic characterization of pectic polygalacturonate 4-α-galacturonosyltransferase solubilized from pollen tube of *Petunia axillaris* using pyridylaminated oligogalacturonates as substrates. *Plant Physiol* 130:374–379.
 36. Diesperger H, Müller CR, Sandermann H, Jr (1974) Rapid isolation of a plant microsomal fraction by Mg²⁺-precipitation. *FEBS Lett* 43:155–158.
 37. Lord JM (1987) Isolation of endoplasmic reticulum: general principles, enzymatic markers, and endoplasmic reticulum-bound polysomes. *Methods Enzymol* 148:576–584.
 38. Hodges TK, Leonard RT (1974) Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. *Methods Enzymol* 32:392–406.
 39. Orłowski M, Meister A (1963) γ-Glutamyl-p-nitroanilide: a new convenient substrate for determination and study of L- and D-γ-glutamyltranspeptidase activities. *Biochim Biophys Acta* 73:679–681.