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GLYCOLIPIDS AND GLYCOPROTEINS FORMED FROM UDP-GALACTOSE BY PEA MEMBRANES

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Key Word Index—*Pisum sativum*; Leguminosae; pea membranes; galactolipids; galactoprotein; galactan; biosynthesis.

Abstract—Pea membranes were incubated with UDP-[^{14}C]galactose and sequentially extracted with lipid solvents and 2 % sodium dodecyl sulfate (SDS). At least three-quarters of the products were SDS-soluble. All fractions contained some [^{14}C]glucose, indicating the presence of an active epimerase which, however, could be inhibited by ADP-ribose. The chloroform–methanol extract contained mainly neutral galactosyl lipids and a small amount of dolicyl monophosphoryl glucose. The chloroform–methanol–water extract contained trace amounts of lipid-linked galactosyl oligosaccharide with properties comparable to polyisoprenyl pyrophosphoryl derivatives. Polyacrylamide gel electrophoresis of SDS-soluble products indicated the formation of both immobile and mobile components with similar size distribution (Sepharose CL-6B). The mobile component only was susceptible to hydrolysis by protease. Periodate oxidation analysis of SDS-soluble and -insoluble products indicated that they were composed mainly of 1 → 6 galactosyl residues, i.e. as in many arabinogalactan proteins and arabinogalactans.

INTRODUCTION

Galactose is a ubiquitous constituent of the polysaccharides found in all higher plants, including pea [1, 2], but little is known about the mechanism for its incorporation into these products. There is evidence that UDP-galactose can be utilized by mung bean membrane preparations to form polymeric products [3] containing galactose units with 1,4-β-linkages [4]. Membrane preparations from rye grass incorporate galactose from UDP-galactose into polymers which may be partially 1,6-linked [5]. No conclusive data are available on the MW and linkage distribution of such products. The present study examines the range of products formed from UDP-galactose by membranes prepared from growing regions of etiolated pea stems. Such membranes form neutral lipids (glycerides and steryl derivatives) from UDP-galactose [6] but there is no information on other products that may be synthesized in this system. Pea stems have been shown many times to contain polysaccharides that are rich in galactose [2] and living pea stems readily incorporated ^{14}C from supplied sucrose into galactose-containing polymers [7].

Most of the galactose found in polymeric products in higher plants is present in *O*-linked galactoprotein [8] or arabinogalactans and their derivatives [1, 8, 9]. The latter occur in two main classes. The arabino-4-galactans are linear polymers of galactopyranose units with 1,4-β-linkage which are substituted to variable degree with 1,3 or 1,5-linked side chains of arabinofuranose residues. They are buffer-insoluble and constituents of pectic and hemicellulose fractions of cell walls. In contrast, the arabino-3,6-galactans are complex polysaccharides which are highly branched, buffer-soluble and distributed widely in plants where they are found both intracellularly and extracellularly [9, 10]. The proportion of 1,3-β- and 1,6-β-linkages differs between plant sources, as does the arabinose:galactose ratio and the kinds of arabinosyl linkages. These arabinogalactans may be linked in part to hydroxyproline residues in proteins to form glycoproteins or proteoglycans. The linkage is with a terminal galactosyl residue, which distinguishes these products from insoluble hydroxyproline-rich wall proteins where the galactosyl linkages are labile to alkali (*O*-serine) and hydroxyproline is linked to arabinose.

This paper examines the products formed by pea membranes from UDP-galactose with particular attention to the synthesis of potential lipid intermediates, galactoprotein and products with characteristics resembling arabinogalactans.

RESULTS AND DISCUSSION

Incorporation of UDP-[^{14}C]galactose into various products

After incubation for 2 min with UDP-[^{14}C]galactose in a standard reaction medium and sequential fractionation of products into chloroform–methanol (1:1), chloroform–methanol–water (10:10:3), 2 % (w/v) SDS and insoluble residue, most of the radioactivity was found in the SDS-soluble fraction (Table 1). Some [^{14}C]glucose was present in all fractions, indicating that UDP-glucose 4-epimerase (EC 5.1.3.2) was present and active [11].

Relatively high concentrations of ADP-ribose, which inhibit this epimerase activity in bacterial [12], and rye grass [5] membranes, also reduced total incorporation into all of the pea membrane-generated fractions, particularly those which had the highest glucose content (chloroform–methanol-soluble and SDS-insoluble). At a concentration of 100 μM ADP-ribose and above, epimerization to UDP-glucose and formation of glucose-containing products was almost eliminated (Table 1).

Table 1. Incorporation of ^{14}C from UDP-[^{14}C]galactose into various fractions: effect of ADP-ribose

ADP-ribose	C/M-soluble	C/M/W-soluble	SDS-soluble	Insoluble
		Incorporation (cpm × 10^{-3}/fraction)		
0	2.2	1.2	28.6	6.6
50	2.3	1.3	28.8	3.8
100	3.2	1.8	31.6	3.6
1000	2.4	1.4	25.1	1.5
5000	1.5	1.0	23.4	1.1
		[^{14}C]galactose/[^{14}C]glucose Ratio		
0	0.67	0.84	0.87	0.36
50	0.76	0.87	0.94	0.57
100	0.85	0.93	0.98	0.85
1000	0.86	0.93	0.99	0.90
5000	0.90	0.94	0.99	0.91

Conditions were the same as those of the standard assay (Experimental) except that ADP-ribose was added as indicated. Each fraction was hydrolysed with acid and the hydrolysate was subjected to paper chromatography with solvent B. The areas corresponding to galactose and glucose were counted and the ratio of galactose to glucose was calculated.

C/M, chloroform–methanol; C/M/W, chloroform–methanol–water.

Subsequent experiments were all conducted in the presence of 100 μM ADP-ribose.

The chloroform–methanol extract was subjected to DEAE-Sepharose (acetate type) chromatography. Elution profiles showed two types of lipids corresponding to neutral glycolipid (90% of label) and moderately charged glycolipid which was eluted with 5 mM ammonium formate in the lipid solvent. Each fraction was further subjected to silica gel thin-layer chromatography. The neutral glycolipid showed a chromatographic mobility similar to that of authentic digalactosyldiglyceride, sterylglycoside and monogalactosyldiglyceride [6] and yielded no free sugar on mild acid hydrolysis. Hydrolysis with strong acid (1 M trifluoroacetic acid) yielded mostly labeled galactose and some glucose. The moderately charged glycolipid co-chromatographed with dolichyl monophosphoryl glucose. The sugar moiety was very susceptible to acid hydrolysis, i.e., in 0.01 M trifluoroacetic acid (pH 2) at 100°, all of the radioactivity became water soluble in less than 10 min. It chromatographed (solvent B) with glucose only. Accordingly, the glucolipid is probably dolichyl monophosphoryl glucose.

The relatively low yield of products soluble in chloroform–methanol–water (10:10:3) was chromatographed on DEAE-Sepharose to yield mainly (90%) highly-charged lipid and a small amount of neutral lipid (Fig. 1). This neutral lipid has not been further studied. The charged component was only partially (30–40%) susceptible to hydrolysis by mild acid (pH 2, 100°, 10 min). Paper chromatography of this hydrolysate (solvent A) yielded [^{14}C]galactose, which probably derived from UDP-galactose, but also labeled non-mobile (origin) material. The latter chromatographed in the V_0 of a Bio-Gel P-2 column and, upon hydrolysis with 2 M trifluoroacetic, was found to contain [^{14}C]galactose as the only labeled sugar. It appears to be a lipid-linked galactosyl oligosaccharide. A product with similar properties was reported [5] formed from UDP-galactose by ryegrass membranes. Whether or not this lipid-linked galactosyl oligosaccharide is a precursor for non-lipid products is speculative at this time.

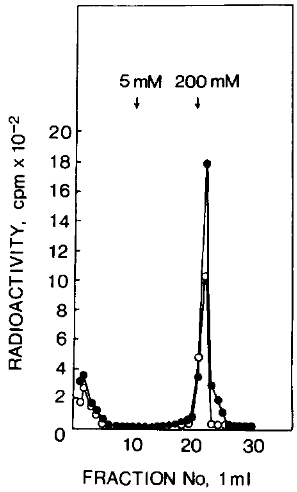

Fig. 1. DEAE-Sepharose chromatography of the chloroform–methanol–water (10:10:3) extract before and after mild acid hydrolysis. Open symbols show the mild acid hydrolysate and closed symbols the original pattern. The column was eluted with chloroform–methanol–water (10:10:3) ± ammonium formate at the concentrations indicated.

The charged glycolipid fraction which was not hydrolysed by mild acid also contained [^{14}C]galactose only upon hydrolysis with strong acid (2 M TFA, 100°, 2 hr) or alkali (0.5 N KOH, 100°, 6 hr). It cannot have been a pyrophosphate derivative but could represent a 'phytoglycolipid' [13] such as phytosphingolipid. Attempts to separate the readily hydrolysed lipid-linked galactosyl oligosaccharide from the acid-resistant charged lipid on the basis of charge or size have been unsuccessful.

SDS-soluble products were washed with acetone and fractionated on Sepharose CL-6B in 1 M NaOH before and after treatment with protease. The profiles obtained are shown in Fig. 2. The untreated products included a labeled V_0 component and others with mobilities equivalent to a range of dextran standards with MW from 10^4 to 5×10^5. Extended (72 hr) treatment with protease resulted in the generation of a small amount of products with relatively low apparent MW in a range up to about 2×10^4. These were produced at the expense of all size ranges of the original products.

Non-lipid products which were SDS-soluble and -insoluble were subjected to Smith degradation and, as shown in Fig. 3, each fraction produced glycerol as the major product, indicating the predominance of $1 \rightarrow 6$ linkages. Some threitol and galactose were also present, suggesting $1 \rightarrow 4$ and $1 \rightarrow 3$ linkages, respectively.

Kinetics and requirements for galactose incorporation into non-lipid products

Radioactivity continued to accumulate in products soluble in chloroform–methanol for 20–30 min, in products soluble in chloroform–methanol–water for 10 min

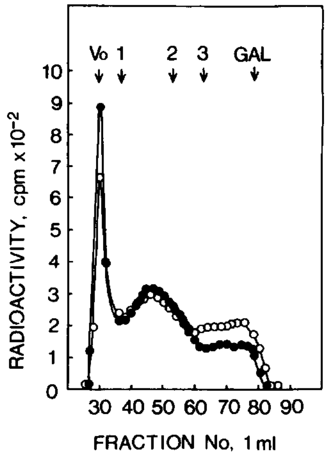

Fig. 2. Gel filtration of the SDS-soluble products before and after protease treatment on Sepharose CL-6B. Open symbols show the protease-treated products and closed symbols without the treatment. Arrows represent the elution position of standards: V_0, Blue dextran; 1, dextran T-500 (MW 500000); 2, dextran T-40 (MW 40000); 3, dextran T-10 (MW 10400); GAL, galactose.

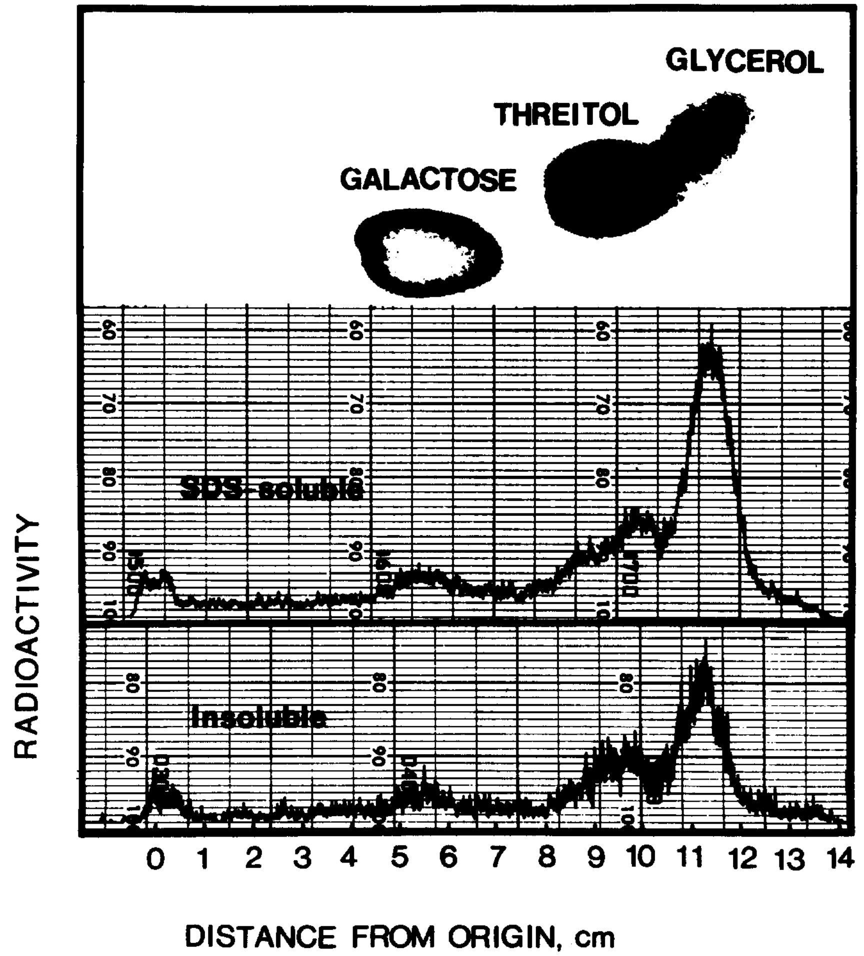

Fig. 3. Paper chromatography of the product by Smith degradation of non-lipid products. The experimental procedure is described in the Experimental.

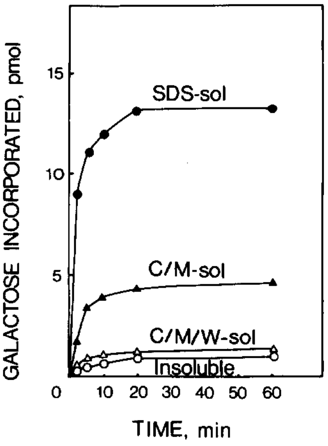

Fig. 4. Time course of incorporation of galactose from UDP-galactose into various fractions by pea membrane preparations. The lipid fractions were shown as chloroform–methanol (1:1) soluble products (C/M) and chloroform–methanol–water (10:10:3) soluble products (C/M/W).

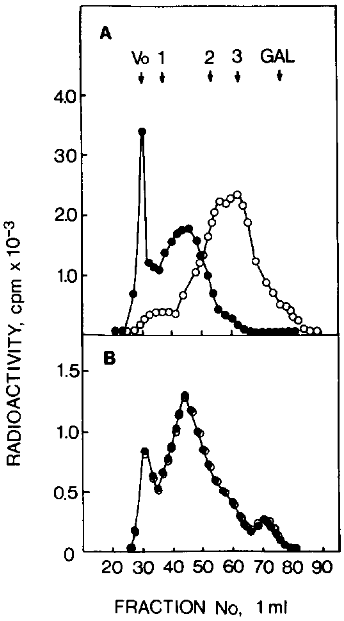

Fig. 5. Gel filtration on Sepharose CL-6B of electrophoretically-mobile [^{14}C]galactosyl component (Panel A) and non-mobile fraction (Panel B) of SDS-soluble fraction before and after protease treatment. Open symbols show the protease-treated component and closed symbols without the treatment. Arrows representative of standards were the same as used in Fig. 2.

and in SDS-soluble and -insoluble products for 20 min (Fig. 4). There was no suggestion of turnover in any fraction. Lineweaver–Burk plots of initial velocities of the formation of SDS-soluble and -insoluble products from UDP-galactose yielded K_m values (5 and 10 μM, respectively) and V_{max} values (400 and 140 pmol/2 min/mg protein respectively). These values should be regarded as 'apparent' since it is probable that they are composites for the synthesis of more than one product in each fraction (see next section). They are relatively very low compared to published values for incorporation of UDP-glucose into water-insoluble products by pea membranes, suggesting that the transgalactosylases responsible must have a relatively high affinity for their substrate and acceptor. Maximal incorporation from UDP-[^{14}C]galactose was attained with an optimum of pH 7.0 for the SDS-soluble products in HEPES–KOH buffer and pH 8.0 for the SDS-insoluble products. At the higher pH there was more [^{14}C]glucose found in these products because of the activation of UDP-glucose 4-epimerase. When Mg^{2+} or Mn^{2+} was added to the reaction mixture, the initial rate of incorporation was highest with Mn^{2+} in the SDS-soluble products and with Mg^{2+} in the SDS-insoluble products. The addition of other sugar nucleotides unlabelled to the reaction systems, e.g. 100 μM UDP-arabinose, UDP-*N*-acetyl glucosamine and GDP-mannose, had no effect on or inhibited incorporation of [^{14}C]galactose from UDP-[^{14}C]galactose.

Characterization of galactosyl protein isolated from SDS-soluble products

When the SDS-soluble products were subjected to SDS-acrylamide gel electrophoresis, a small part of the ^{14}C (3 %) was mobile for a short distance (2–4 cm) into the gel. The mobile components fractionated on Sepharose CL-6B to yield two main peaks, one with very high apparent MW (V_0) and the other with an approximate average MW of 130 000. Treatment with protease greatly reduced the size of all of these products to components with an approximate MW of 12 000 (Fig. 5A). Thus, these products appear to be glycoproteins rather than proteoglycans. Treatment with alkali (2 M NaOH at 25° for 12 hr) did not change the original MW distribution on Sepharose CL-6B. It appears that the electrophoretically-mobile SDS-soluble products contain galactosylated protein which is not O-linked through serine or threonine [8].

The electrophoretically-immobile fraction showed a gel-filtration pattern on Sepharose CL-6B (peaks in the V_0 and 130 000 MW range) which was remarkably similar to that of the electrophoretically-mobile components (Fig. 5B). Since treatment of the immobile products with protease for 72 hr did not reduce their size, they may be unconjugated polysaccharides. When the mobile and immobile fractions were separately subjected to Smith degradation, glycerol, galactose and threitol were produced in proportions of 8.3:1.2:1.0 and 8.3:0.45:1.0, respectively. This indicates that both subfractions of the SDS-soluble products contain mainly 1 → 6 linked galactose units, with smaller amounts of 1 → 4 and 1 → 3 linkages.

These results demonstrate that pea membranes are capable of incorporating galactose from UDP-galactose into a variety of products besides neutral lipids, including protease-digestible glycoprotein and electrophoretically-immobile SDS-soluble and -insoluble polysaccharide(s). All of these products contain 1,6-linked galactose units primarily, and in this respect resemble certain arabinogalactans and arabinogalactan proteins, e.g. as recently described in pollen of timothy grass [14]. Fractionation studies (Figs 2 and 5) indicate that the SDS-soluble components are polydisperse with a MW range mostly above 100000, i.e. similar to arabino-galactans from *Gladiolus* pollen [10]. Finally, the protease-digestible product was resistant to hydrolysis in alkali which is consistent with the properties of hydroxyproline-linked galactose residues, as found in arabinogalactan protein [15, 16]. Further studies are in progress to investigate the possibility that pea membranes are able to incorporate arabinose as well as galactose into products resembling arabinogalactans.

EXPERIMENTAL

Materials. UDP-[^{14}C]galactose (302 mCi/mmol), Aquasol and Protosol were obtained from New England Nuclear. Unlabeled sugar nucleotides, bovine serum albumin, dextran T-500, and protein MW (bovine plasma albumin, ovalbumin, lysozyme) were from Sigma. Dextran T-40 and T-10, DEAE-Sepharose CL-6B, and Sepharose CL-6B were from Pharmacia, Pronase (protease preparation from *Streptomyces griseus*) from Boehringer Mannheim and silica gel-60 thin layer (0.2 mm) sheets from EM laboratories.

General methods. TLC for lipid-linked sugars was performed on silica gel-60 plates (0.2 mm) in $CHCl_3$–MeOH–H_2O (65:25:4). PC for sugars was performed with Whatman 3 MM paper in *n*-PrOH–EtOAc–H_2O 3:2:1 (solvent A) and *n*-BuOH–Pyr–H_2O, 6:4:3 (solvent B). Radioactivity on paper was detected by a Packard radiochromatogram scanner, model 7200; protein was determined according to the method of ref. [17], carbohydrate by the phenol–H_2SO_4 method [18] and radioactivity with a Beckman CPM-100 liquid scintillation spectrometer, using Aquasol for aq. samples and toluene scintillator for lipid samples and paper strips (1 × 3 cm).

Assay for galactose incorporation into lipid- and non-lipid products. Total membrane mixtures were prepared from apical growing regions (10 mm) of week-old etiolated pea stems by grinding and differential centrifugation, as described earlier [6]. Membrane aliquots (0.6–1.2 mg of protein) in 300 μl of 0.1 M HEPES–KOH buffer (pH 6.8) containing 1 mM dithiothreitol, 0.1 % bovine serum albumin and 0.4 M sucrose were incubated at 25° for 2 min with 10 μl (1.65 nmol) of UDP-[^{14}C]galactose (302 mCi/mmol) containing 3 μmol $MnCl_2$ and 30 μmol ADP-ribose in a total vol. of 310 μl.

Reactions were routinely terminated by adding 1.5 ml of $CHCl_3$–MeOH–H_2O (2:2:1) to give a mixture of $CHCl_3$–MeOH–H_2O (1:1:1). This resulted in a total lipid extract in two phases and an insoluble residue. The phases were separated by centrifugation and the lower phase was removed. The upper layer and interface were washed with another 1 ml of $CHCl_3$ and the two lower phases were combined to provide the $CHCl_3$–MeOH (2:1) fraction. The upper phase plus interface was mixed with 4 ml of MeOH and centrifuged. The pellet was then washed ×4 with ice-cold 70 % MeOH before being extracted for 2 hr at −20° in order to provide the $CHCl_3$–MeOH–H_2O (10:10:3) fraction. Both lipid fractions were evaporated to dryness in a vial in order to determine radioactivity.

For mild hydrolysis of intact glycones from phospholipids, lipid extracts were treated with 0.01 M TFA in 50 % *n*-PrOH in a sealed tube at 100° for 10 min. For complete hydrolysis, glycolipids were treated with 2 M TFA. After hydrolysis, the reaction mixtures were evaporated to dryness and the products dissolved in a small vol. of H_2O for further analysis.

In order to measure the incorporation of [^{14}C]galactose into non-lipid products, the residue obtained after the extraction of lipids was extracted twice with 1 ml of 2 % SDS in 4 mM Tris–HCl (pH 6.8) and 10 % glycerol at 85° for 20 min. This produced fractions referred to as SDS-soluble and -insoluble. The SDS-soluble fraction was made 80 % with Me_2CO and centrifuged. The pellet and the Me_2CO-washed SDS-insoluble fraction were suspended in 90 % TFA at room temp. for 18 hr. The suspension was then diluted with H_2O to a final concn of 20 % acid and hydrolysed in a sealed tube at 100° for 2 hr. The reaction mixture was evaporated to dryness to remove TFA and submitted to PC with solvent B in order to determine the ^{14}C-sugar content.

Fractionation of lipids by DEAE-Sepharose. The lipid extract was added to a DEAE-Sepharose (acetate type, CL-6B) column (0.5 × 2 cm) which was eluted with 10 ml of $CHCl_3$–MeOH–H_2O (10:10:3), to collect neutral lipids, the same solvent containing 5 mM ammonium formate to collect monophosphoryl glycolipids and the solvent containing 200 mM ammonium formate for more highly charged glycolipids [6]. Each 1 ml extract was collected in a fraction collector, and ^{14}C was assayed in aliquots. The elution profiles of authentic phosphoryl polyprenyl sugars from DEAE-Sepharose were exactly the same as those from DEAE-cellulose.

Acrylamide electrophoresis of SDS-soluble products was performed on 10 % gels containing 0.1 % SDS [19]. The gel tracks were sliced with a razor into 0.5 cm fractions. Slices were placed in scintillation vials in 3 % Protosol with toluene scintillator and the mixture was incubated at 37° for 24 hr in order to extract completely the radioactivity from the gel [20].

Smith degradation. A sample of oligosaccharide or polysaccharide was incubated at 4° for 72–96 hr in the dark with 30 μmol of Na metaperiodate in 100 mM NaOAc, pH 5.6, in a total vol. of 1 ml [21]. The reaction mixture was then reduced by the addition of 100 μmol $NaBH_4$. After 2 hr the reaction was stopped by the addition of TFA to a final concn of 2 M and the sample was hydrolysed by heating at 100° for 2 hr. The mixture was concd to dryness to remove TFA and deionised with Rexyn 101 (H^+). Borate was removed by repeated addition of MeOH and evaporation (× 4) and the sample was subjected to PC with solvent A.

Enzymatic hydrolysis and Sepharose fractionation. For removal of SDS, each sample was washed × 5 with 80 % Me_2CO. Samples were suspended in 20 mM NaPi buffer (pH 7) containing 0.2 % NaN_3, and incubated for 72 hr at 37° with 5 mg of protease preparation (Pronase, 70 PUK/mg). The preparation was then boiled, reduced in vol. and applied to the appropriate fractionation procedure in order to evaluate the effects. Samples with or without protease treatment were suspended in 1 M NaOH and applied to a column (1 × 100 cm) of Sepharose CL-6B and eluted with 0.1 M NaOH.

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