POTATO TUBER GLUCOSYL TRANSFERASES: PARTIAL CHARACTERIZATION OF THE SOLUBILIZED ENZYMES

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1. Introduction

Evidence indicates that the synthesis of protein bound α-1,4 glucosidic linkages in potato tuber is associated with membranous structures. A two step reaction sequence [1–3] was shown to occur in a potato tuber particulate preparation:

UDP–Glc + Acceptor protein → Acceptor protein–Glc + UDP (1)

Acceptor protein–Glc + UDP–Glc (ADP–Glc or Glc-1-P) → Acceptor protein–Glc–$(Glc)_n$ + UDP (ADP or P_i) (2)

The enzyme catalyzing reaction (1), named UDP–Glc:protein transglucosylase I [1], has been shown to be specific for UDP–Glc, with K_m 4×10^{-6} M. It transfers only 1 glucosyl moiety to the aminoacyl residue on the endogenous acceptor protein [2]. Glucosidic linkages are established using the product of reaction (1), once UDP–Glc or ADP–Glc or Glc-1-P is present in the reaction mixtures at 10^{-3} M [2,3], with glucosyl transferases of the same enzymatic preparation.

The property shown by various enzymes involved in protein-bound glucan synthesis, consisting in their being membrane-linked, complicates the separation of the different enzymes or acceptor proteins from other membrane-bound proteins. To understand the individual biosynthetic steps that ultimately lead to formation of protein-bound glucan in more detail, it is necessary to solubilize some of the glucosyl transferases involved in this pathway.

Here we report on the solubilization of potato tuber particulate enzymatic activities, by increasing the ionic strength of the medium. Partial characterization of the solubilized enzymes catalyzing reaction (1) and (2) and their products, is also reported.

This work is dedicated to Professor Carlos E. Cardini on his 70th birthday

2. Materials and methods

2.1. *Preparation of membranes and solubilization of enzymes*

Membranes from potato tuber (particulate preparation) were obtained as in [1]. For enzyme solubilization, membranes (40–80 mg protein/ml) were treated with Tris–HCl buffer 0.1 M (pH 7.4) containing 5 mM 2-mercaptoethanol, for 48–72 h at 0°C. Clear supernatants were obtained after a 120 min centrifugation at 140 000 × *g* and used as source of enzymes (solubilized preparation).

2.2. *Enzyme assays*

Transfer of glucose from UDP-[^{14}C]Glc to endogenous protein acceptor (reaction (1)) was measured as incorporation of [^{14}C]glucose into 10% hot trichloroacetic acid insoluble material. Unless otherwise specified, the standard incubation mixture contained, in 50 μl total vol.: 75 000 cpm UDP-[^{14}C]Glc (284 Ci/mol), 6–10 mM $MnCl_2$ and 60–300 μg solubilized membrane protein. Incubations were at 30°C for 20 min. The addition of 4 mM unlabeled UDP–Glc, ADP–Glc or Glc-1-P to the standard incubation mixture allowed the estimation of reaction (2). In the latter case, incubations were for 240 min.

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2.3. *Other procedures*

Conditions for proteolytic and amylolytic treatments were as in [1,4]. Alkaline borohydride reduction was done as in [2]. Protein was determined according to [5]. SDS/urea/polyacrylamide gel electrophoresis was performed on a 10% slab gel in Tris–bicine buffer (pH 8.3) [6,7]. The gels were stained for protein with Coomassie brilliant blue.

Chromatography and electrophoresis were carried out on Whatman no. 1 paper, with the following solvents: (A) 5% formic acid (pH 2.5); (B) 0.25 M sodium carbonate buffer (pH 9.2); (C) 0.1 M sodium molybdate buffer (pH 5.0); (D) butanol:pyridine:water (6:4:3, by vol.). Reducing substances were located with the alkaline silver reagent [8], and radioactivity in the paper strips was scanned on a Packard radiochromatogram scanner, model 7201.

3. Results and discussion

The results of estimating glucosyl transfer activities of the solubilized preparation to endogenous acceptor proteins or to added polysaccharide primers are shown in table 1. As already reported for the particulate preparation [1] reaction (1) is specific for UDP–Glc, in the absence as well as in the presence of glycogen. Exogenous polysaccharide could not compete with the limiting levels of endogenous acceptor at 4 μM UDP-[^{14}C]Glc (table 1, expt II). It is very likely that the enzyme catalyzing reaction (1) is also specific for the endogenous acceptor and that a classical synthetase from the solubilized preparation accounts for the formation of labeled methanol-insoluble material.

The terms 'classical synthetase' or 'classical phosphorylase' are used here to describe an enzyme which catalyzed glucose transfer between UDP–Glc or ADP–Glc or Glc-1-P as donors and exogenous polysaccharide as acceptor. Reaction (2) was clearly observed in the presence of unlabeled 4 mM UDP–Glc or ADP–Glc or Glc-1-P (table 1, expt I). Sometimes, radioactive methanol-insoluble–trichloroacetic acid-soluble material can be found in addition to the acid-insoluble products. This may be due to the formation of long α-1,4 glucosidic chains bound to protein or generation of trichloroacetic acid-soluble fragments by endogenous hydrolyzing enzymes [9].

Classical synthetases and phosphorylases were also noted when glycogen was included as exogenous acceptor (table 1). To date, there is not enough available information to decide whether these classical elongating enzymes are the same that catalyze reaction (2) with the product of reaction (1) as acceptor.

Table 1
Solubilization of glucosyl transfer activities

	Glucose donor	Radioactivity (cpm) incorporation in	
		Trichloroacetic acid precipitate	Methanol precipitate
Expt I			
With endogenous acceptor	UDP–[^{14}C]Glc 4 μM	1799	15
	UDP–[^{14}C]Glc 4 mM	1900	937
	UDP–[^{14}C]Glc 4 μM + ADP–Glc 4 mM	2277	3270
	UDP–[^{14}C]Glc 4 μM + Glc-1-P 4 mM	5636	3115
Expt II			
With exogenous acceptor	UDP–[^{14}C]Glc 4 μM	2203	1203
	ADP–[^{14}C]Glc 4 μM	200	10 703
	[^{14}C]Glc-1-P 4 μM	208	4008

Membranes were extracted as in section 2. Incubations were performed with 250 μg protein at 30°C for 4 h. The radioactive material precipitated with 10% trichloroacetic acid or with methanol after precipitation with trichloroacetic acid was measured as in [4]. Glycogen (400 μg) was added as the exogenous acceptor in expt II. ADP–[^{14}C]-Glc (87 000 cpm) 0.2 nmol, or [^{14}C]Glc-1-P (45 000 cpm) 0.2 nmol, were included in the incubation mixtures instead of UDP–[^{14}C]Glc, as indicated

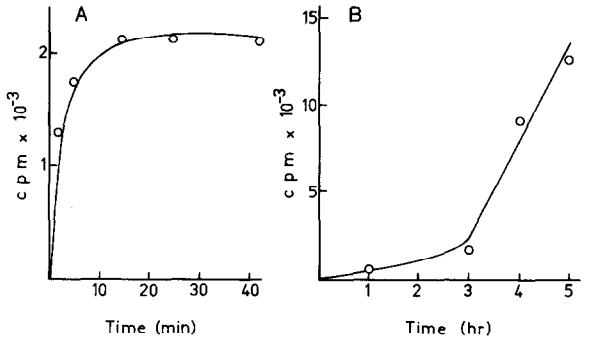

Fig.1. Time course of reaction (1) and (2): (A) 90 μg protein was present in reaction (1); (B) 240 μg protein in reaction (2). The assays were as in section 2.

Some enzymatic activities engaged in the biosynthesis of protein-bound α-1,4 glucosidic linkages were extracted from membranes of potato tuber by increasing ionic strength. This property suggests that the active proteins are presumably bound by predominantly polar interactions to the proteins or the lipids in the membrane [10].

The dependence on time and on the amount of protein of both solubilized reactions with endogenous acceptors were studied. Fig.1 illustrates the time course for both reactions. Since reaction (1) very rapidly reaches a plateau value for [^{14}C]glucosyl protein formation, an incubation time of 20 min was chosen for the standard enzyme test; for reaction (2) an incubation time of 240 min was taken. Under these conditions both reactions show a linear dependence on the amount of solubilized protein added in the range shown in fig.2A,B.

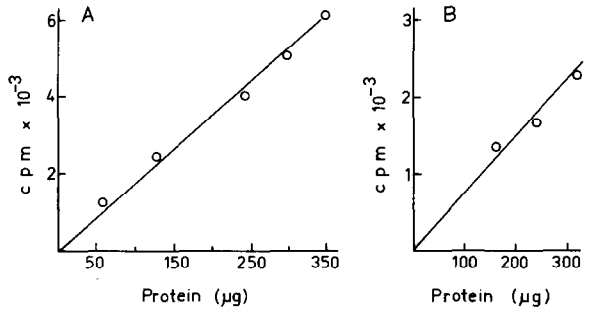

Fig.2. Dependence on protein concentration of reaction (1) and (2): (A) formation of (acceptor protein)–[^{14}C]Glc was measured in 20 min; (B) incubation time was 180 min, with the addition of 4 mM unlabeled UDP–Glc to the standard incubation mixture. Other experimental conditions, as outlined in section 2.

3.1. *Characterization of the 'in vitro' reaction products*

Reaction (1) product obtained under the standard assay conditions was submitted to exhaustive proteolytic digestion with pronase (type VII, Sigma) at pH 8.0. No further precipitation of the radioactivity was obtained upon addition of 10% hot trichloroacetic acid. Experiments were carried out with the soluble labeled pronase digest to detect formation of a linkage of the type peptide–[^{14}C]glucose. Chromatography of the pronase digest on a column of Sephadex G-15 resulted in the inclusion of the label. The pooled radioactive fractions were subjected to electrophoresis on Whatman no. 1 paper in solvents A and B. A radioactive peak migrating to the cathodic end was found on the electrophoretogram at pH 2.5 and one migrating to the anodic end, at pH 9.2. Alkaline borohydride reduction studies of the compound present in the radioactive peak eluted from the electrophoretogram in solvent A, indicate the formation of [^{14}C]sorbitol, identified by paper electrophoresis in solvent C. These studies conclusively proved that glucose in the glucopeptide is involved in *O*-glucosidic linkage with the amino acid of the endogenous protein acceptor.

β-Amylase had no effect on reaction (1) product, while glucoamylase released almost all the label as [^{14}C]glucose, as judged by paper chromatography in solvent D. However, β-amylase digestion carried out on reaction (2) product resulted in the release of 87% of the incorporated radioactivity as [^{14}C]maltose. Glucoamylase and α-amylase treatments yielded [^{14}C]glucose and labeled glucose and maltose, respectively. These experiments pointed on the occurrence of α-1,4 glucosidic linkages in reaction (2) product.

Attempts were made to separate the enzymes

Table 2
Glucose transfer activity in the partially purified preparation (DEAE-step)

	Radioactivity (cpm) incorporation in	
	Trichloroacetic acid precipitate	Methanol precipitate
With endogenous acceptor	2803	35
With exogenous polysaccharide	3062	42

Incubations for estimating reaction (1) were performed with 18 μg protein for 60 min. Other experimental conditions as outlined in section 2. Glycogen (200 μg) was added as exogenous polysaccharide

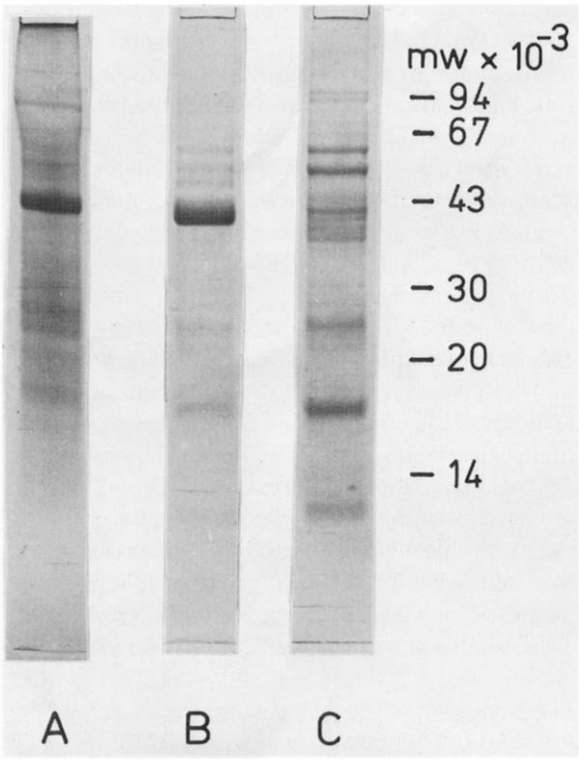

Fig.3. SDS/urea/polyacrylamide gel electrophoretic patterns: (A) potato tuber particulate preparation (80 μg protein); (B) solubilized preparation (80 μg protein); (C) partially purified UDP–Glc:protein transglucosylase I (DEAE-step) (40 μg protein). Numbers on the right side of the figure indicate app. M_r-values estimated from the mobility of marker proteins in a parallel track.

catalyzing both reactions with endogenous acceptors. The solubilized preparation, containing 10–20 mg of protein was chromatographed on a DEAE-cellulose column. The UDP–Glc:protein transglucosylase I activity was eluted by ~0.2 M KCl. Highly active enzyme fractions were pooled and used as partially purified enzyme (DEAE-step). Other glucosyl transfer activities were also retained on the column, but they eluted as a bulk by higher [KCl]. No glucosyl transfer activity to exogenous polysaccharide was detected in the partially purified enzyme (DEAE-step) (table 2). Chromatography on DEAE succeeded in separating enzyme catalyzing reaction (1) from classical synthetases and phosphorylase, but failed in separating it from the endogenous acceptor. The enzyme catalyzing reaction (1) was purified 25-fold over the solubilized preparation which corresponds to ⩾50-fold purification with respect to the particulate preparation. Fig.3 shows the electrophoretic profiles of different enzymatic fractions. The starting material (particulate preparation) profile as well as the solubilized preparation profile are dominated by one major band (38 000 M_r) (fig.3A,B) and multiple minor bands. The 15 000, 56 000 and 63 000 M_r protein bands are most concentrated in the DEAE-fraction containing only UDP–Glc:protein transglucosylase I activity (fig.3C).

Further purification of the enzymes catalyzing both reactions will be required to resolve the question of whether the endogenous acceptor and the enzymes are similar or distinct proteins.

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