Distinct isoforms of ADPglucose pyrophosphatase and ADPglucose pyrophosphorylase occur in the suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.)

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Abstract The intracellular localizations of ADPglucose pyrophosphatase (AGPPase) and ADPglucose pyrophosphorylase (AGPase) have been studied using protoplasts prepared from suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). Subcellular fractionation studies revealed that all the AGPPase present in the protoplasts is associated with amyloplasts, whereas more than 60% of AGPase is in the extraplastidial compartment. Immunoblots of amyloplast- and extraplastid-enriched extracts further confirmed that AGPase is located mainly outside the amyloplast. Experiments carried out to identify possible different isoforms of AGPPase in the amyloplast revealed the presence of soluble and starch granule-bound isoforms. We thus propose that ADPglucose levels linked to starch biosynthesis in sycamore cells are controlled by enzymatic reactions catalyzing the synthesis and breakdown of ADPglucose, which take place both inside and outside the amyloplast.

Key words: Amyloplast; Gluconeogenesis; Phosphodiesterase; Starch

1. Introduction

The currently prevailing hypothetical view concerning starch biosynthesis assumes that intracellular levels of ADPglucose (ADPG) linked to starch production are exclusively controlled by ADPG pyrophosphorylase (AGPase) [1]. However, the recent finding of widely distributed phosphodiesterase activities catalyzing the hydrolytic breakdown of ADPG has led to the proposal that the intracellular level of this nucleotide sugar is determined by the balance between ADPG-synthesizing and -degrading enzyme activities [2]. The suggested role for ADPG pyrophosphatase (AGPPase) is to modulate the accumulation of ADPG by diverting it into alternative pathways in response to biochemical needs. The fact that AGPPase is regulated by factors other than substrate concentration and its activities are inversely correlated to the content of starch in the plant tissues may pinpoint the reaction catalyzed by this novel enzyme as a potential limiting step of the gluconeogenic process.

The hypothesis that the intracellular levels of ADPG linked to starch biosynthesis are determined by the reactions catalyzed by both AGPase and AGPPase begs the question as to which subcellular compartment(s) is/are occupied by these enzymes. So far, the majority of studies dealing with starch biosynthesis in heterotrophic cells have been tightly connected with the sole localization of AGPase in the amyloplast [1,3–5]. In contrast, recent investigations have shown that AGPase is principally located in the cytosolic compartment of cells of cereal endosperms [6–9] and tomato pericarp [10]. On the other hand, it must be pointed out that preliminary analyses obtained in this laboratory have revealed the presence of both intra- and extraplastidial isoforms of AGPPase in plant tissues [2].

In order to thoroughly explore the subcellular localization of both AGPPase and AGPase in starch-forming heterotrophic cells, suspension-cultured cells of sycamore were chosen as a model system. A previously established standard method, which enables the isolation of intact, pure, and metabolically active amyloplasts free from contaminating starch granules [11,12], is believed to have obvious advantages for the examination of the enzymatic machineries as well as the regulatory mechanisms operating in the amyloplast. In the present study it was established that a major portion of the AGPase activity was present in the extraplastidial compartment, whereas different isoforms of AGPPase were located in the amyloplast. Based on these observations, we have further advanced our previously proposed mechanism according to which the levels of gluconeogenic intermediates linked to starch formation are determined by tightly interconnected enzymatic reactions taking place both inside and outside the amyloplast [13].

2. Materials and methods

2.1. Protoplast and amyloplast isolation

Protoplasts from the cultured cells of sycamore (*Acer pseudoplatanus* L.) were obtained as previously described by Frehner et al. [11]. Highly purified and intact amyloplasts free from contaminating starch granules were isolated according to the Percoll step gradient centrifugation method described by Pozueta-Romero et al. [12].

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Abbreviations: ADH, alcohol dehydrogenase; ADPG, ADPglucose; AGPase, ADPG pyrophosphorylase; AGPPase, ADPG pyrophosphatase (or ADPG phosphodiesterase); G1P, glucose 1-phosphate; 6PGDH, 6-phosphogluconate dehydrogenase; NiR, nitrite reductase; PFK(PPi), pyrophosphate–fructose-6-phosphate 1 phosphotransferase; PPase, alkaline pyrophosphatase; SS, sucrose synthase; TPI, triose-P-isomerase; UGPase, UDPglucose pyrophosphorylase

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2.2. Enzyme assays

Samples subjected to enzyme assays were desalted by ultrafiltration on Centricon YM-10 (Amicon, Bedford, MA, USA) and resuspended in a solution containing 50 mM HEPES (pH 7.5), 1 mM dithiothreitol and 5 mM $MgCl_2$. Except for the case of AGPPase and sucrose synthase (SS), methods employed to assay enzyme activities are given as follows: name of the enzyme assayed, EC number and the corresponding reference. AGPase and UDPglucose pyrophosphorylase (UGPase), EC 2.7.7.27 and EC 2.7.7.9, respectively [14]; pyrophosphate–fructose-6-phosphate 1 phosphotransferase (PFK(PPi)), EC 2.7.1.90 [15]; alkaline pyrophosphatase (PPase), EC 3.6.1.1 [16]; alcohol dehydrogenase (ADH), EC 1.1.1.1 [17]; α-mannosidase, EC 3.2.1.24 [18]; 6-phosphogluconate dehydrogenase (6PGDH), EC 1.1.1.44 [19]; nitrite reductase (NiR), EC 1.7.7.1 [20]; and triose-P-isomerase (TPI), EC 5.3.1.1 [21].

SS, EC 2.4.1.13, was assayed in the sucrolytic direction by the chromatographic determination of ADPG and UDPG as described by Rodríguez-López et al. [2]. The reaction mixture contained 50 mM HEPES (pH 7.0), protein extract, 500 mM sucrose and 5 mM of the corresponding nucleoside diphosphate in a total volume of 50 μl. All assays were run with blanks without nucleoside diphosphate. After 5 min of incubation, the reaction was stopped by boiling the reaction mixture in a dry bath for 1 min. The mixture was centrifuged at $20\,000\times g$ for 5 min and the supernatant was saved. AGPPase activities were measured by determining the glucose 1-phosphate (G1P) and AMP produced upon ADPG breakdown, as described by Rodríguez-López et al. [2].

Protein content was measured by the method of Bradford using a Bio-Rad prepared reagent and bovine γ-globulin as standard.

The unit (U) is defined as the amount of enzyme which catalyzes the formation of 1 μmol of product per minute.

2.3. Solubilization of starch granule-bound AGPPase

Starch granules (14.4 mg) were obtained from amyloplasts lysed with 1% Triton X-100 and subsequently centrifuged at $100\,000\times g$ for 30 min. The pellet was resuspended in 1 ml of 50 mM HEPES (pH 7.0) buffer and incubated with α-amylase (35 U/mg starch) and amyloglucosidase (1 U/mg starch) for 60 min at room temperature with stirring [22]. The digested starch suspension was then centrifuged at $100\,000\times g$ and the supernatant was removed for subsequent analysis.

2.4. Gel filtration chromatography

Protein samples were subjected to gel filtration on a Superdex 200 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) pre-equilibrated with MES (pH 6.0) plus NaCl 150 mM. The elution was carried out with the same buffer at a flow rate of 0.2 ml/min and 150 ml fractions were collected. The native molecular masses of AGPPase isoforms were determined from a plot of K_{av} (partition coefficient) versus log molecular mass of the following protein standards: bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), ovalbumin (45 kDa), myoglobin (17 kDa) and vitamin B-12 (1.3 kDa).

2.5. Western blot analyses

Protein samples were separated on 10% SDS–PAGE and transferred to nitrocellulose filters. Immunodetection of sycamore AGPase, UGPase and NiR was carried out using the antisera raised against the tomato AGPase [23], potato UGPase [24] and *Cucurbita pepo* NiR [25].

3. Results and discussion

3.1. Quality of the amyloplast preparations

Employing the discontinuous Percoll gradient centrifugation technique described by Pozueta-Romero et al. [12], highly purified, intact amyloplasts from the suspension-cultured sycamore cells were obtained. Judging by the activities of soluble plastid markers, PPase and NiR, 1% of the amyloplasts originally present in the protoplast lysates were obtained in the final amyloplast preparation (Table 1). The activities of the contaminating cytosolic markers, i.e. ADH, PFK(PPi), SS [26] and UGPase [27] as well as the vacuolar marker, i.e. α-mannosidase, were found to be almost undetectable; overall results thus show that our preparations can be satisfactorily used to analyze the enzymatic machineries existing in the amyloplast.

Comparisons of enzyme activities in fractions obtained at the end of the preparation with those in the initial protoplast lysate as well as in the differential centrifugation step guaranteed no loss of activity during the preparation for any of the enzymes analyzed in this work (Table 1).

Table 1
Purity, yield and content of some enzymes during isolation of amyloplasts from suspension-cultured cells of sycamore

Enzyme	Protoplast lysate	Differential centrifugation		Stepped Percoll gradient centrifugation		
	Activity (mU/g fw)	Sediment (% of lysate)	Recovery range (%)	Amyloplast activity (mU/g fw)	Amyloplast (% of lysate)	Recovery range (%)
Target enzymes						
AGPPase	4.9 ± 2.0	40.5 ± 11.3	108 ± 22	0.05 ± 0.02	1.00 ± 0.17	120 ± 39
AGPase	37 ± 11	18.2 ± 5.7	86 ± 9	0.15 ± 0.05	0.43 ± 0.23	136 ± 21
Amyloplast markers						
PPase	1285 ± 364	44.8 ± 6.4	106 ± 17	12.8 ± 3.05	1.0 ± 0.2	82 ± 14
NiR	128 ± 4.7	33.1 ± 8.9	109 ± 29	1.3 ± 0.65	1.0 ± 0.5	140 ± 33
Intra/extraplastidial enzymes						
6PGDH	199 ± 103	25.2 ± 8.7	95 ± 22	1.03 ± 0.68	0.5 ± 0.2	79 ± 16
TPI	1189 ± 628	22.9 ± 18.7	99 ± 19	6.82 ± 5.30	0.5 ± 0.3	157 ± 23
Contaminant markers						
Cytosolic:						
ADH	756 ± 466	7.4 ± 3.9	103 ± 17	0.04 ± 0.01	0.006+0.01	110 ± 31
PFK(PPi)	516 ± 89	20.4 ± 6.8	101 ± 26	0.23 ± 0.10	0.044 ± 0.02	100 ± 29
UGPase	109 ± 24	22.4 ± 6.2	116 ± 7	0.07 ± 0.02	0.060 ± 0.02	121 ± 11
SS (ADPG synthesis)	76 ± 23	6.6 ± 2.8	92 ± 11	b.d.l.	–	–
SS (UDPG synthesis)	217 ± 70	11.2 ± 2.3	90 ± 9	b.d.l.	–	–
Vacuolar:						
α-Mannosidase	96 ± 22	7.9 ± 2.6	94 ± 8	0.05 ± 0.03	0.048 ± 0.02	83 ± 26

Protoplast lysates were subjected to differential centrifugation and the resulting pellets were separated on a stepped Percoll density gradient (cf. Fig. 1). Activities of the protoplast lysates are expressed as the total activity of the lysate fractions divided by the fresh weight (fw) of the original cells without correction for protoplast yield. Data represent means ± S.E.M. of up to eight independent experiments.
b.d.l. = below detection limit.

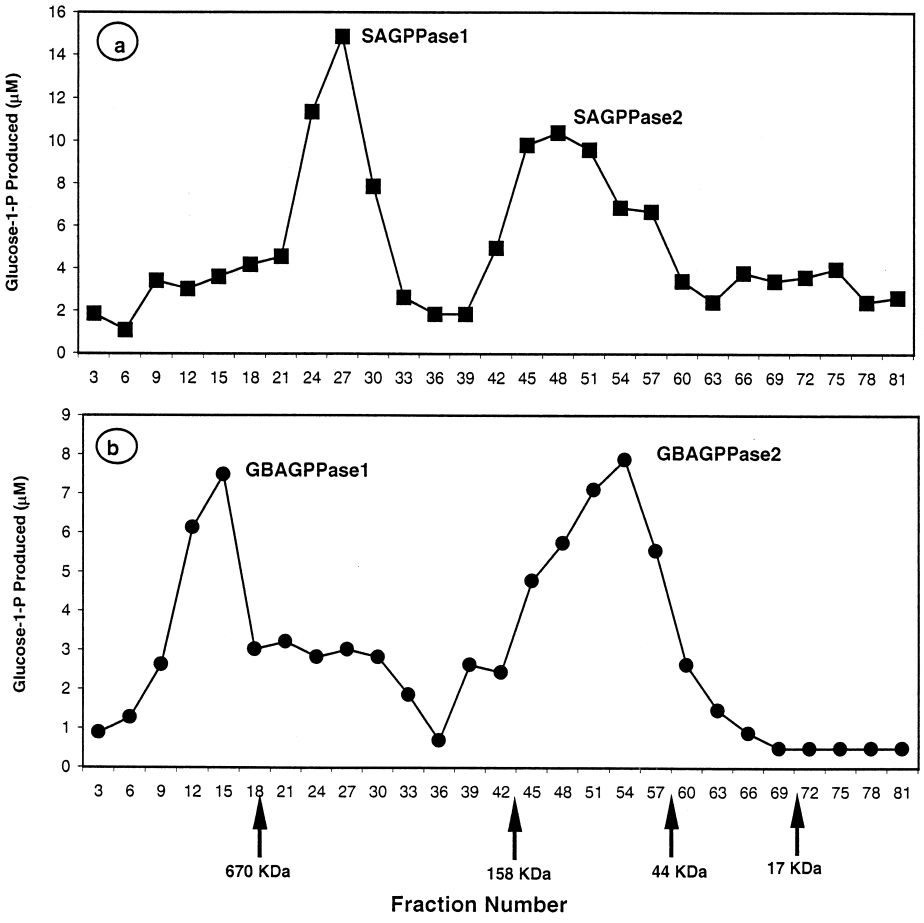

Fig. 1. Partial purification of different isoforms of AGPPase using gel filtration chromatography. Disrupted amyloplasts were subjected to 100 000 × *g*. A: The supernatant was directly applied to gel filtration (11.5 mU of AGPPase). B: The 100 000 × *g* supernatant obtained after solubilization of 9.5 mU of AGPPase (see Section 2) was applied to gel filtration.

3.2. Presence of different isoforms of AGPPase in amyloplasts

Consistent with our previous report [2], results presented in Table 1 reveal that 1.0% of the AGPPase activity initially present in the protoplast lysate was recovered in the final amyloplast preparations. This value is significantly different (95% confidence limits) from the values of recovery of cytosolic marker enzymes. With a confidence limit of 95%, there is no significant difference between the AGPPase yields in the amyloplast preparations and those of plastidial markers. Therefore, it can be reasonably estimated that all the AGPPase activity in the sycamore protoplasts is associated with amyloplasts.

We also investigated the spatial distribution of AGPPase in the amyloplasts. Towards this end, disrupted amyloplasts were subjected to ultracentrifugation, and enzymatic activities analyzed in both the soluble and particulate fractions. Results in Table 2 clearly show the presence of both soluble and particulate AGPPase activities, the latter being not extractable using 1% Triton X-100. Assuming that the particulate activity is bound to the starch granules, solubilization using starch hydrolyzing enzymes was attempted. As shown in Table 2, the particulate AGPPase activity was found to be completely solubilized after treatment with α-amylase and amyloglucosidase, indicating that it is bound to the starch granule.

Extracts containing soluble AGPPase activities and those solubilized from starch granules were separately subjected to gel filtration chromatography. As presented in Fig. 1a, we could detect ca. 350 kDa and ca. 75 kDa soluble AGPPase isoforms (designated SAGPPase1 and SAGPPase2, respectively). As shown in Fig. 1b, ca. 800 kDa and ca. 50 kDa granule-bound AGPPase isoforms (designated GBAGPPase1 and GBAGPPase2, respectively) were detected.

Table 2
Spatial distribution of AGPPase inside the amyloplast

Treatment	Activity (% of initial AGPPase activity)	
	Sediment	Supernatant
(1) Control (freeze and thaw)	85.5	14.5
(2) +Triton X-100	92.5	7.5
(3) +α-Amylase and amyloglucosidase	n.d.	100.0

Amyloplasts (0.25 mU of AGPPase in a volume of 100 μl) were treated as indicated and centrifuged at 100 000 × *g*.
n.d. = not detectable.

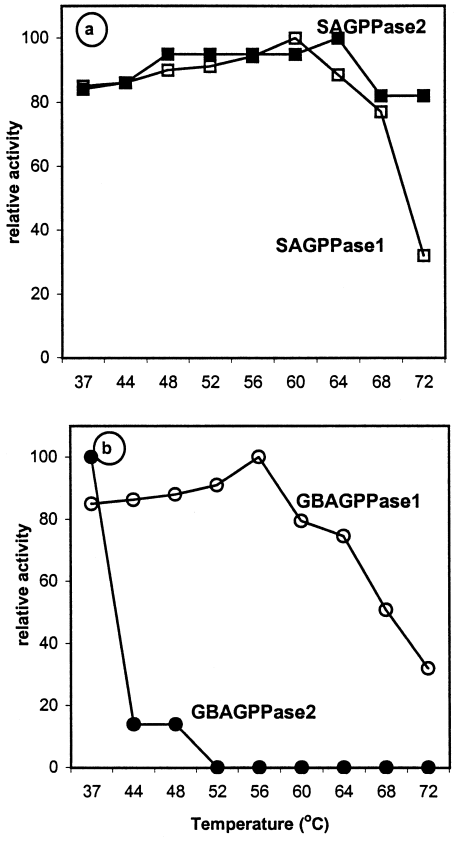

Fig. 2. Temperature–activity curve of partially purified (a) SAGPPase1 and SAGPPase2, and (b) GBAGPPase1 and GBAGPPase2 obtained from the gel filtration chromatography presented in Fig. 1.

Partially purified isoforms of AGPPase were used for further biochemical characterizations. As presented in Fig. 2a,b, analogous to the soluble AGPPase isolated from barley leaves [2], SAGPPase1, SAGPPase2 and GBAGPPase1 were found to be resistant to high temperature regimes, whereas GBAGPPase2 was heat-labile.

SAGPPase1 has a broad optimum pH occurring between pH 4.0 and pH 7.5, whose activities dramatically decline at basic pH values (Fig. 3a). In sharp contrast, GBAGPPase2 is totally inactive at acid pH values and has a broad optimum pH occurring between pH 7.0 and 10.0 (Fig. 3b). GBAGPPase 1 and SAGPPase2 have optimal activities at pH 7.0 and 9.0, respectively (Fig. 3a,b).

Overall results thus strongly indicate the existence of various molecular entities catalyzing the hydrolytic breakdown of ADPG in the amyloplast. These observations have been further confirmed by our presently ongoing research dealing with the purification and biochemical characterization of both soluble and starch granule-bound AGPPase using tomato pericarp and barley leaves (Zandueta-Criado et al., in preparation).

In vitro analyses of the starch synthase activities in amyloplast preparations incubated with or without purified AGPPase strongly indicated that both AGPPase and starch synthase compete for ADPG [2]. Since there have been reported soluble and granule-bound isoforms of starch synthase [28], the present finding showing the presence of soluble and granule-bound isoforms of AGPPase inside the amyloplast further strengthens the view that AGPPase may control starch biosynthesis by modulating the magnitude of substrate availability for starch synthases. Needless to say, the use of transgenic plants with altered AGPPase activities will provide us with a decisive tool to test the validity of this challenging mechanism.

3.3. Less than 40% of the total AGPase activity in sycamore cells is plastidial

AGPase has escaped the attention of previous studies examining the subcellular distribution of enzymes in suspension-cultured sycamore cells [11]. However, later studies showing the G1P-dependent starch biosynthesis in amyloplasts isolated from sycamore cells [29] strongly indicated that these organelles do contain AGPase.

Results shown in Table 1 reveal that 0.4% of the AGPase activity initially existing in the protoplast lysate was present in the amyloplast preparations. This value is significantly different (95% confidence limits) from those of cytosolic and plastidial marker enzymes. Furthermore, this value is comparable to the yields of enzymes which occur both inside and outside the amyloplast, i.e. TPI and 6PGDH [30,31]. It should also be emphasized that, as shown by Rodríguez-López et al. [2], some AGPase activities measured may be ascribable to artifactual determinations made using extracts which contain AGPPase, since measurements of both AGPase and AGPPase are based on the determination of G1P derived from the ADPG breakdown. Therefore, the results of the present study

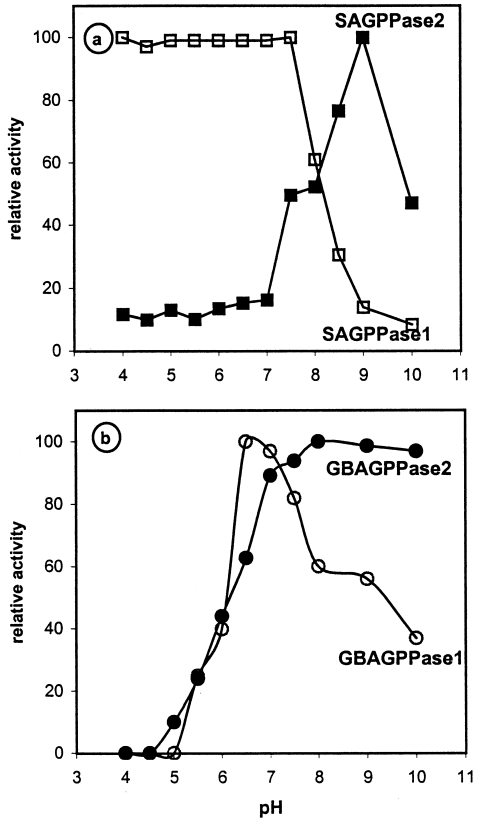

Fig. 3. pH–activity curve of partially purified (a) SAGPPase1 and SAGPPase2, and (b) GBAGPPase1 and GBAGPPase2 obtained from the gel filtration chromatography represented in Fig. 1. 50 mM sodium acetate–acetic acid (pH 3.0–5.5), 50 mM MES–NaOH (pH 5.5–6.5), 50 mM HEPES–NaOH (pH 6.5–8.0) and 50 mM Tris–HCl (pH 8.0–9.0) buffers were used.

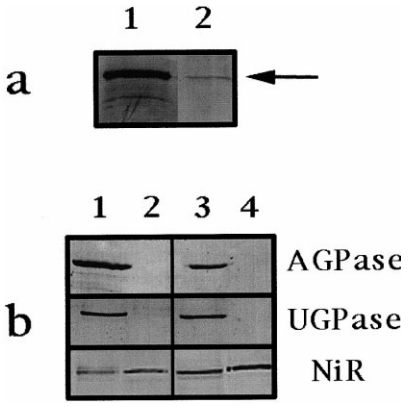

Fig. 4. Extraplastidial localization of AGPase in sycamore cultured cells. a: Western blot of tomato pericarp extracts (lane 1) and sycamore amyloplasts (lane 2, 6 mU of PPase). b: Western blot of extracts of the supernatant of the differential centrifugation step (lanes 1 and 3) and of the amyloplast preparation (lanes 2 and 4). The samples correspond to two different preparations. Doses of PPase loaded onto each lane were: lanes 1 and 2: 2.7 mU; lanes 3 and 4: 3.0 mU.

may in fact indicate that less than 0.4% of the AGPase initially existing in the protoplast lysate was present in the amyloplast preparations. Based on the apparent AGPase activities recovered in the amyloplast preparations, and following the equation developed by Thorbjornsen et al. [7], it is estimated that less than 40% of the total AGPase activity in the sycamore cells is associated with amyloplasts.

In attempting to further explore the possibility that there exists a minor plastidial AGPase and an abundant extraplastidial AGPase in the sycamore protoplasts, Western blot analyses using antibodies raised against the tomato AGPase were performed after SDS–PAGE separation of proteins from the amyloplast preparations and from the extraplastid-enriched supernatant fraction of the differential centrifugation step. In parallel, Western blot analyses using antibodies raised against the cytosolic UGPase marker and the plastidial NiR marker were performed. In every case, the electrophoresed samples were adjusted to equal amounts of soluble plastidial marker enzymes. It is suspected that, if AGPase is exclusively localized in the plastidial compartment, immunodecoration of this enzyme should reveal bands of the same intensity in samples corresponding to the amyloplast preparations and those enriched with extraplastidial components. However, as shown in Fig. 4, essentially similar to the case of Western blots using the UGPase antibodies, immunoblots of AGPase revealed bands whose intensities were significantly stronger in the extraplastidial samples compared to the amyloplast samples. In contrast, immunoblots of NiR revealed bands of the same intensity in the amyloplast and extraplastidial samples.

Overall results thus confirm that, essentially as in the case of AGPase from developing endosperms of maize and barley [6,7], AGPPase from the cultured cells of sycamore is principally localized outside the amyloplast.

3.4. Additional remarks

Although the various types of AGPPase found in the sycamore protoplasts are shown to be located in the amyloplast, previous studies on barley endosperms have shown the occurrence of both intra- and extraplastidial phosphodiesterases catalyzing the hydrolytic breakdown of different nucleotide sugars [2]. Therefore, the results of the present study indicate the possible existence of different types of phosphodiesterases catalyzing the hydrolysis of nucleotide sugars in the plant cell. Since the major role of nucleotide sugars is believed to serve as donors of glycosyl residues for numerous reactions [32], the possibility that nucleotide sugar phosphodiesterases may be engaged in controlling the levels of nucleotide sugars linked to the biosynthesis of starch, cell wall polysaccharides, glycoproteins and glycolipids cannot be ruled out.

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