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ORIGINAL ARTICLE

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ADP-glucose pyrophosphorylase from wheat endosperm. Purification and characterization of an enzyme with novel regulatory properties

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Abstract ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) was purified and characterized from two wheat (*Triticum aestivum* L.) tissues: leaf and endosperm. The leaf enzyme, purified over 1,300-fold, was found to be a heterotetramer composed of subunits of 51 and 54 kDa and possessing regulatory properties typical of AGPases from photosynthetic tissues, being mainly regulated by 3-phosphoglycerate (activator; $A_{0.5} = 0.01$ mM) and orthophosphate (inhibitor; $I_{0.5} = 0.2$ mM). Conversely, the enzyme from wheat endosperm was insensitive to activation by 3-phosphoglycerate and other metabolites. It was, however, inhibited by orthophosphate ($I_{0.5} = 0.7$ mM), ADP ($I_{0.5} = 3.2$ mM) and fructose-1,6-bisphosphate ($I_{0.5} = 1.5$ mM). All of these inhibitory actions were reversed by 3-phosphoglycerate and fructose-6-phosphate. The endosperm enzyme was found to be a heterotetramer composed of subunits of 52 and 53 kDa, which were recognized by antiserum raised to spinach leaf AGPase. The results suggest that wheat endosperm AGPase possesses distinctive regulatory properties that are relevant in vivo.

Keywords ADP-glucose pyrophosphorylase · Endosperm · Enzyme regulation · Starch synthesis · *Triticum* (AGPase)

Abbreviations ADP-Glc: ADP-glucose · AGPase: ADP-glucose pyrophosphorylase · Fru-1,6-bisP: fructose-1,6-bisphosphate · Fru-6-P: fructose-6-phospahte · Glc-1-P: glucose-1-phosphate · PEG: polyethylene glycol · PEP: phospho*enol*pyruvate · 3PGA: 3-phosphoglycerate

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Introduction

ADP-glucose pyrophosphorylase (AGPase; EC 2.7.7.27) catalyzes the synthesis of ADP-glucose (ADP-Glc) and PPi from glucose-1-phosphate and ATP, being the regulatory enzyme in the pathway for glycogen and starch synthesis in bacteria and plants, respectively (Preiss 1999). AGPase is generally modulated by allosteric effectors and is tetrameric in structure. A common characteristic of the enzyme regulators is that they are key intermediates of the major pathway of carbon assimilation in the respective organism (Sivak and Preiss 1998).

AGPase from cells performing oxygenic photosynthesis is allosterically regulated by 3-phosphoglycerate (3PGA; activator) and Pi (inhibitor). There is strong evidence that regulation by these metabolites is relevant in vivo, the 3PGA/Pi ratio being a main signal determining starch synthesis within the chloroplast (Sivak and Preiss 1998). Conversely, evidence concerning regulation of AGPase from non-photosynthetic tissues of plants is less clear-cut. AGPases from maize and rice endosperm, potato tuber, and cassava root are regulated by 3PGA and Pi (Sivak and Preiss 1998). However, AGPases from pea embryos (Hylton and Smith 1992), barley endosperm (Kleczkowski et al. 1993; Rudi et al. 1997) and bean cotyledons (Weber et al. 1995) were found to be insensitive to regulation.

Explanations for the lack of 3PGA activation of several AGPases have been proposed, e.g. proteolysis of the maize endosperm enzyme (Plaxton and Preiss 1987). It is also possible that AGPases from some reserve tissues may have distinctive properties. Moreover, other possibilities have been suggested that challenge the involvement of the enzyme in starch synthesis in amyloplasts of reserve tissues. These "alternative" hypo theses include: (i) an alternative pathway avoiding AGPase (Akazawa et al. 1991), and (ii) a cytosolic AGPase insensitive to regulation (Kleczkowski 1996; Thorbjørnsen et al. 1996). In both models, ADP-Glc is

synthesized in the cytosol and the regulation of AGPase is not relevant in the metabolic pathway. As indicated in several reviews (Okita 1992; ap Rees 1995; Sivak and Preiss 1998), most of the experimental evidence contradicts the alternative hypotheses. Still, regulation of the pathway of polysaccharide accumulation in reserve tissues of higher plants merits further investigation.

In this paper we describe the purification and characterization of wheat endosperm AGPase, demonstrating that it has regulatory properties different from those AGPases studied so far. The properties of the endosperm and leaf enzymes are compared and discussed in a physiological context.

Materials and methods

Plant material and reagents

Wheat (*Triticum aestivum* L. cv. Pampa) was grown in the field. Developing seeds (28 days post-anthesis) were detached, frozen in liquid nitrogen and stored at –80 °C until use. Leaves were obtained from plants in the pre-anthesis period and used immediately.

Radiochemicals were from DuPont NEN. All reagents were of the highest quality.

Enzyme and protein assays

AGPase activity was determined in the direction of ADP-Glc pyrophosphorolysis (assay A) or synthesis (assay B), under identical conditions to those described elsewhere (Iglesias et al. 1993, 1994). One unit of enzyme activity is defined as the amount of AGPase synthesizing 1 μmol product/min under the respective conditions.

Protein concentration was determined after Peterson (1977), using BSA as the standard.

Kinetic and structural studies

$S_{0.5}$, $A_{0.5}$, and $I_{0.5}$, the concentrations giving 50% maximal activity, activation, and inhibition, respectively, and Hill coefficients (n_H) were calculated after Brooks (1992). All kinetic parameters are means of at least two determinations and are reproducible within ±10%.

SDS-PAGE was performed according to Laemmli (1970). Proteins were transferred onto nitrocellulose membranes (Burnette 1981) and immunodetected with rabbit anti-spinach leaf AGPase IgG. The molecular mass of native AGPase was estimated using a Superose 12 column, as described elsewhere (Gómez Casati et al. 2000).

Purification of AGPase

All steps were performed at 4 °C and enzyme activity monitored using assay A.

Purification of the leaf enzyme

Wheat leaves were homogenized using a mortar and pestle in buffer A [25 mM Mops-NaOH (pH 7.5), 5 mM Pi, 1 mM EDTA, 10 mM 2-mercaptoethanol (2-ME)]. Following centrifugation (12,000 *g*, 15 min) the supernatant was heated for 5 min at 60 °C in a water bath. After placing on ice for 5 min, the sample was centrifuged and the supernatant adsorbed onto DEAE-cellulose. The enzyme was eluted with a linear gradient [5 bed volumes buffer A against 5 bed volumes buffer containing 25 mM Mops-KOH (pH 6.0), 5 mM Pi, 1 mM EDTA, 10 mM 2-ME, 400 mM KCl]. Following concentration (through Amicon PM-30), the sample was dialyzed against buffer B [50 mM Hepes-KOH (pH 7.0), 5 mM Pi, 5% sucrose, 1 mM EDTA, 2 mM 2-ME].

The sample was adsorbed onto QAE-cellulose and then eluted using a linear KCl gradient (0–1 M) in buffer B. After concentration, 2 M K-phosphate (pH 7.5) was added to bring the sample to 1 M Pi, followed by adsorption onto a Phenyl agarose column equilibrated with 1 M K-phosphate (pH 7.5) and 2 mM 2-ME. The enzyme was eluted stepwise with decreasing concentrations of Pi (500, 200, 100 and 5 mM), then concentrated and finally chromatographed on Superose 12 [in 25 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 5 mM Pi, 10 mM 2-ME, 100 mM KCl]. The purified enzyme was concentrated and stored at –20 °C, being stable under these conditions for at least 3 months.

Purification of the endosperm enzyme

Wheat seeds were thawed (3 ml/g) in buffer C [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM K-phosphate, 0.1 mM EDTA, 10 mM 2-ME) supplemented with proteases-inhibitor (0.1 mM tosyl phenylalanyl chloromethyl ketone, 0.1 mM leupeptin and 1 mM phenylmethylsulfonyl fluoride), and homogenized with an Omnimixer. Following centrifugation (15 min, 12,000 *g*), crude extract was brought to 10% (w/v) polyethylene glycol (PEG) 8000, centrifuged again and the supernatant brought to 20% (w/v) PEG. The resulting precipitate was resuspended in buffer C.

To the sample, crystalline ammonium sulfate was added to 30% saturation and the mixture centrifuged. The supernatant was brought to 60 °C within 5 min, kept for 4 min at this temperature, cooled for 5 min on ice, and centrifuged. Following dialysis against buffer C, the sample was applied to Mono Q HR5/5. The enzyme was eluted with a linear KCl gradient (0–500 mM) in buffer C, concentrated (through Centricon PM-30) and then chromatographed on Superose 12 using buffer C containing 100 mM KCl. The purified enzyme was concentrated and stored at –20 °C, thus retaining 80% activity for 1 month.

Results

Purification of AGPase from leaves and endosperm of wheat

In crude extracts from wheat leaves, AGPase was activated nearly 4-fold by 3PGA and remained stable after 5 min heating at 60 °C. Conversely, in wheat endosperm extracts, enzyme activity was insensitive to 3PGA and the heat treatment inactivated it by more than 95%. Indeed, in wheat endosperm extracts AGPase was unstable even at 4 °C, with loss of 50% activity within 6–8 h. Enzyme stability was increased by ammonium sulfate, as a supernatant of 30% saturation retained 50% of the activity after heat treatment. Also, partial protection from the heating step was afforded by PEG (10–20%) fractionation. However, the best procedure for high activity recovery after heating was to perform PEG fractionation followed by ammonium sulfate (30% saturation) fractionation. This allowed more than 70% activity to be retained and stabilized the endosperm AGPase to further purification steps.

We optimized two purification protocols for the AGPases from wheat leaves and endosperm (Tables 1 and 2, respectively). The leaf enzyme was purified 1,384-fold to a specific activity of 58.5 U/mg protein (final recovery 30%) (Table 1), whereas the endosperm AGPase

was purified 12-fold (specific activity 2.4 U/mg protein), with a 26% final yield (Table 2). Although steps comprising PEG and ammonium sulfate fractionation, and heat treatment led to relatively low purification (Table 2), they were critical in stabilizing the enzyme.

Remarkably, the amount of AGPase activity was different in each tissue, being higher in endosperm (4.4 U/g fresh tissue) than in leaves (0.3 U/g fresh tissue). This is expected for a soluble enzyme, since the endosperm tissue is more dehydrated than the leaf. Figure 1 shows an SDS-PAGE analysis of the purified leaf (Fig. 1a) and endosperm (Fig. 1b) enzymes. Two protein bands were revealed for the purified leaf AGPase, with molecular masses of 51 and 54 kDa (Fig. 1a). The molecular mass of the native enzyme was determined to be 210 kDa (data not shown). These results agree with those for the spinach leaf enzyme, showing a heterotetrameric ($\alpha_2\beta_2$) structure (Morell et al. 1987).

Concerning the AGPase from endosperm, silver staining detected a wide protein band in the range 52–53 kDa (Fig. 1b), which was visualized by immunoblot using anti-spinach leaf AGPase (Fig. 1c). These results indicate that the wheat endosperm enzyme is composed of two subunits, with slightly different molecular masses, as found for the potato tuber enzyme (50 and 51 kDa subunits, Okita et al. 1990). Supporting this, Ainsworth et al. (1995) isolated a full-length cDNA clone representing the large subunit of wheat endosperm AGPase: the deduced protein contained 522 amino acids (57.8 kDa), including a transit peptide (62 amino acids, 6.5 kDa). Size-exclusion chromatography revealed that the native wheat endosperm enzyme is a heterotetramer of 220 kDa (data not shown).

Table 1 Purification of AGPase from wheat (*Triticum aestivum*) leaves. Data correspond to a typical purification of 200 g of fresh leaves

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	1,925	83	0.04	1	100
Heat treatment	253	101	0.4	9	121
DEAE-cellulose	24	61	2.6	59	74
QAE-cellulose	13	54	4.2	98	65
Phenyl agarose	4	37	9.8	228	45
Superose 12	0.4	24	58.5	1,384	29

Table 2 Purification of AGPase from wheat endosperm. Data correspond to a typical purification from 84 g of seeds

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude extract	1,878	370	0.20	1	100
PEG fractionation	1,385	286	0.21	1.1	77
Ammonium sulfate	1,033	250	0.24	1.2	68
Heat treatment	548	174	0.32	1.6	47
Mono-Q	112	133	1.19	6.0	36
Superose 12	39	95	2.44	12	26

Regulatory properties of the AGPases from leaves and endosperm

To explore the in vivo regulation of wheat AGPases in photosynthetic vs. storage tissues, we analyzed the effect of different metabolites on the purified enzymes. For this study we chose the ADP-Glc synthesis direction of the enzyme reaction for two reasons: (i) it is the physiologically relevant reaction, and (ii) it is more affected by allosteric effectors (Sivak and Preiss 1998). Table 3 shows substantial differences concerning the effect of metabolites on the activity of each of the two enzymes. AMP, fructose-6-phosphate (Fru-6-P), phospho*enol*-pyruvate (PEP) and 3PGA activated the AGPase from

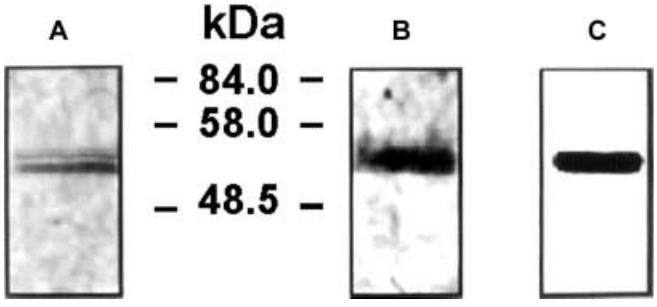

Fig. 1 SDS-PAGE of AGPase purified from leaves (*lane A*) and endosperm (*lane B*) of wheat (*Triticum aestivum*). Numerals indicate molecular masses of the following standards: Fru-6-P kinase (84 kDa), pyruvate kinase (58 kDa), and fumarase (48.5 kDa). *Lane C* shows western blot analysis of the endosperm enzyme after transferring the SDS-PAGE shown in lane B to nitrocellulose, followed by incubation with antiserum raised against spinach leaf AGPase

Table 3 Effect of different metabolites on the activity of AGPase purified from leaves and endosperm of wheat. Activity was assayed in the ADP-Glc synthesis direction (assay B) in the presence of the stated metabolites at 2 mM concentration, using 0.02 µg and 0.5 µg of enzyme purified from wheat leaf or wheat endosperm, respectively. Glc-6-P, pyruvate, NAD(H), and NADP(H) showed no effect on the ADP-Glc synthesis activity of the enzyme

Compound	AGPase activity			
	Leaf		Endosperm	
	nmol/10 min	%[a]	nmol/10 min	%[a]
None	0.30	100	4.10	100
ADP	0.29	97	2.46	60
AMP	0.74	247	3.89	95
Pi	0.03	10	1.02	25
Fru-6-P	1.50	487	4.08	100
Fru-1,6-bisP	0.94	313	1.89	46
PEP	1.39	463	3.61	88
3PGA	3.36	1,120	4.11	100

[a]100% activity is the value corresponding to the assay in the absence of any addition for the respective enzyme from leaves or endosperm

wheat leaves, but showed no effect (or slight inhibition by AMP and PEP) on the endosperm enzyme (Table 3). Also, 2 mM fructose-1,6-bisphosphate (Fru-1,6-bisP) activated the leaf enzyme 3.1-fold, but inhibited the endosperm enzyme by 55%. Pi inhibited both AGPases, although to different extents, and the endosperm enzyme was also inhibited by ADP (Table 3).

In accord with the properties reported for AGPase from photosynthetic cells (Gómez Casati et al. 2000), 3PGA and Pi were the main effectors of the wheat leaf enzyme (Table 3). This is reinforced in Table 4, which shows the kinetics for the activatory/inhibitory effect of different metabolites: the leaf enzyme was inhibited by Pi ($I_{0.5}=0.2$ mM) and activated 11-fold by 3PGA ($A_{0.5}=0.01$ mM), whereas Fru-1,6-bisP, PEP, AMP and Fru-6-P activated the enzyme to a lesser extent and with higher $A_{0.5}$ values. Table 4 also shows an interaction between the inhibitory effect of Pi and the activation by 3PGA, Fru-1,6-bisP and PEP. In the presence of 2 mM Pi, $A_{0.5}$ values increased, with the kinetics becoming cooperative (Table 4). A similar interaction, especially between Pi and 3PGA, has already been reported for other AGPases (Gómez Casati et al. 2000). Contrarily, inhibition by Pi had no effect on activation of the wheat leaf enzyme by AMP and Fru-6-P.

After finding that the endosperm AGPase was insensitive to 3PGA, we determined if this metabolite had any effect on the inhibition by Pi. Figure 2 shows inhibition by Pi ($I_{0.5}=0.7$ mM) and insensitivity of endosperm AGPase to 3PGA alone. Notably, 3PGA reversed the inhibition caused by 2 mM Pi, restoring almost 100% activity at 4 mM (Fig. 2). The interaction between Pi and 3PGA in the regulation of endosperm AGPase showed that increasing concentrations of 3PGA reduced inhibition by Pi (see $I_{0.5}$ values in Table 5). Reciprocally, $A_{0.5}$ and n_H values for 3PGA activation increased as Pi concentration increased (Table 5).

The sensitivity of wheat endosperm AGPase to 3PGA in the presence of Pi prompted us to study the effect of the metabolites tested in Table 3, but in the presence of 2 mM Pi. Under these conditions, ADP and Fru-1,6-bisP further inhibited the enzyme already affected by Pi, whereas Fru-6-P, PEP and AMP activated the enzyme by 1.8-, 1.4-and 1.2-fold, respectively, and 3PGA (2 mM) reversed the inhibition caused by Pi, increasing the enzyme activity 2.5-fold (Table 6). In addition, 3PGA and Fru-6-P were effective in reversing the inhi-

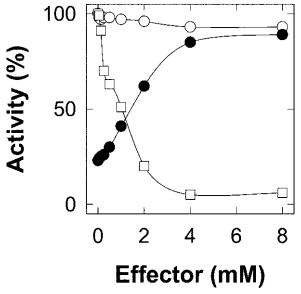

Fig. 2 Effect of Pi and/or 3PGA on the activity of AGPase from wheat endosperm. Enzyme activity was determined in the ADP-Glc synthesis direction (assay B) at the stated concentrations of Pi (*squares*) or 3PGA (*open circles*), or in the presence of 2 mM Pi and different concentrations of 3PGA (*closed circles*). The value of enzyme activity corresponding to 100% was 0.82 μmol·(mg protein)$^{-1}$ min^{-1}

Table 4 Kinetic parameters for allosteric effectors of wheat leaf AGPase. Enzyme activity was assayed in the ADP-Glc synthesis direction (assay B) in the presence of variable concentrations of the corresponding effector. The kinetics of activation by 3PGA, Fru-1,6-bisP, PEP, AMP or Fru-6-P were determined in the absence or in the presence of the inhibitor Pi, as indicated

Compound	$A_{0.5}/I_{0.5}$ (mM)	n_H	Activation (-fold)
Pi	0.2	1.2	
3PGA	0.01	1.0	11
3PGA + 2 mM Pi	1.9	2.3	
Fru-1,6-bisP	0.06	0.9	3.3
Fru-1,6-bisP + 2 mM Pi	3.0	3.1	
PEP	0.2	1.0	4.6
PEP + 2 mM Pi	5.2	1.7	
Fru-6-P	3.6	0.9	4.9
AMP	1.3	1.3	2.4

Table 5 Kinetic parameters for inhibition by Pi and activation by 3PGA of AGPase purified from wheat endosperm. Enzyme activity was assayed in the ADP-Glc synthesis direction (assay B) in the presence of variable concentrations of one effector (Pi or 3PGA), and in the absence or in the presence of a fixed amount (as indicated) of the second effector (3PGA or Pi)

Compound	$A_{0.5}$ /$I_{0.5}$ (mM)	n_H
Pi	0.70	1.3
Pi + 0.25 mM 3PGA	0.91	0.9
Pi + 1 mM 3PGA	1.52	1.1
Pi + 5 mM 3PGA	2.49	1.4
3PGA	n.e[a]	
3PGA + 0.7 mM Pi	0.81	1.0
3PGA + 1.5 mM Pi	1.51	1.4
3PGA + 3 mM Pi	2.52	2.1
3PGA + 5 mM Pi	3.33	2.5

[a]No effect

Table 6 The effect of different metabolites on the activity of AGPase from wheat endosperm after inhibition by Pi. Activity was assayed in the ADP-Glc synthesis direction (assay B) in the presence of 2 mM Pi plus the stated metabolites at 2 mM concentration, using 0.5 μg of purified enzyme per activity assay. Glc-6-P, pyruvate, NAD(H), and NADP(H) showed no effect on the enzyme activity

Compound	ADP-Glc synthesis activity	
	nmol/10 min	%
None	1.30	100
ADP	0.72	55
AMP	1.56	120
Fru-6-P	2.34	180
Fru-1,6-bisP	0.78	60
PEP	1.82	140
3PGA	3.25	250

bition caused by Pi, ADP and/or Fru-1,6-bisP (see below), but AMP and PEP only exhibited a partial effect even at high concentrations (30 mM, data not shown).

Figure 3 illustrates reversal by 3PGA of the inhibition of endosperm AGPase by Pi, ADP and Fru-1,6-bisP, separately or together, and shows interaction between the effectors. ADP and Fru-1,6-bisP inhibited the enzyme ($I_{0.5}$ = 2.7 and 1.5 mM, respectively). Inhibition by 2 mM ADP or by 2 mM Fru-1,6-bisP was reversed by 3PGA (Fig. 3a), which was also effective in reversing inhibition of endosperm AGPase by mixtures of Pi/ADP, Fru-1,6-bisP/ADP, or the three inhibitors together (Fig. 3b). Clearly, enzyme activity is determined by the relative concentration of each allosteric effector in the assay medium.

Fru-6-P reversed inhibition of wheat endosperm AGPase by 0.5 mM Pi or 2 mM ADP, with almost total reversion reached at 5 and 20 mM Fru-6-P, respectively (Fig. 4). However, Fru-6-P only partly reversed inhibition of the enzyme by a mixture of Pi/ADP. The effect of Fru-6-P was also observed in the presence of 3PGA, suggesting that one activator is effective in the presence of the other (Fig. 4).

The kinetics of endosperm AGPase at varying concentrations of glucose-1-phosphate (Glc-1-P) and ATP followed hyperbolic saturation curves for both substrates ($S_{0.5}$ = 0.092 and 0.12 mM, respectively). Identical results were obtained for kinetics performed in the presence of 3PGA. This 3PGA-independent high affinity toward the substrates seems to be a characteristic of the wheat endosperm enzyme, since AGPases from other sources generally exhibit a low affinity for Glc-1-P and ATP in the absence of 3PGA, and require the allosteric activator to reach $S_{0.5}$ values comparable to those found for the endosperm enzyme (Iglesias et al. 1993; Sivak and Preiss 1998). Indeed, kinetics for the substrates, calculated for the wheat leaf enzyme gave values for $S_{0.5}$ and n_H (in parentheses) of 0.45 mM (1.1) for Glc-1-P and 0.73 mM (1.2) for ATP in the absence of 3PGA, whereas in its presence these values were reduced to 0.08 mM (1.0) and 0.22 mM (1.1) for Glc-1-P and ATP, respectively.

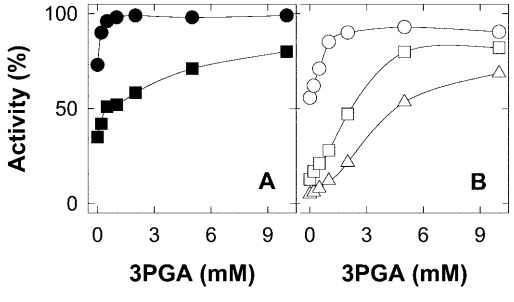

Fig. 3 Reversal by 3PGA of the inhibition of AGPase from wheat endosperm caused by ADP or Fru-1,6-bisP alone (**a**) and by mixtures of these metabolites with Pi (**b**). Activities were determined using assay B, at the stated concentrations of 3PGA, in media containing 2 mM ADP (*closed circles*), 2 mM Fru-1,6-bisP (*closed squares*), 0.5 mM Pi plus 2 mM ADP (*open circles*), 0.5 mM Pi plus 2 mM Fru-1,6-bisP (*open squares*), or 0.5 mM Pi, plus 2 mM ADP and 2 mM Fru-1,6-bisP (*triangles*)

Purified wheat endosperm AGPase as the major active form in the reserve tissue

One possible explanation for the distinctive properties of wheat endosperm AGPase is that extracts from the reserve tissue may contain multiple isoforms of the enzyme. This is particularly important in wheat, which is allohexaploid, and may potentially contain a complex mix of AGPase isoforms in an unknown ratio. The AGPase characterized here could itself be a mixture of isoforms or the only form remaining active throughout purification. To evaluate this, a crude extract of wheat endosperm was analyzed by two high-resolution separation techniques.

Analytical chromatofocusing (pH 4–7) showed only one peak (isoelectric point of 6.1) with AGPase activity, representing nearly 95% of that found in endosperm extracts (Fig. 5a). Isoelectric focusing and immunoblots of the sample also revealed that only one protein (focusing at pH 6.1) was recognized by antiserum to spinach leaf AGPase (Fig. 5B). These results suggest that in endosperm extracts the AGPase exists as one major form, which exhibits regulatory properties [inhibited by Pi ($I_{0.5}$ = 0.75 mM), but insensitive to 3PGA which, however, reversed inhibition by 2 mM Pi ($A_{0.5}$ = 1.5 mM) (Fig. 5, inset)] similar to those of the purified AGPase, thus indicating identity with the pure enzyme described in this work.

Discussion

A wealth of experimental evidence supports a key role for AGPase in starch biosynthesis. In leaves, the enzyme is chloroplastic and is allosterically inhibited by Pi and activated by 3PGA, with the ratio of these metabolites regulating starch synthesis within the chloroplast (Okita 1992; ap Rees 1995; Preiss 1999,). In contrast,

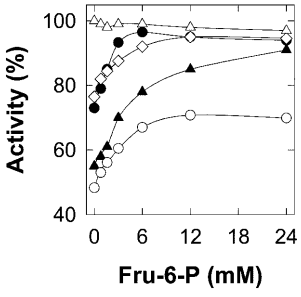

Fig. 4 Reversal by Fru-6-P of the inhibition of AGPase from wheat endosperm. Enzyme activity was determined using assay B, at the different concentrations of Fru-6-P and in the absence of further additions (*open triangles*) or in the presence of: 2 mM ADP (*closed circles*), 1 mM Pi (*closed triangles*), 1 mM Pi plus 2 mM ADP (*open circles*), or 1 mM Pi plus 2 mM 3PGA (*diamonds*)

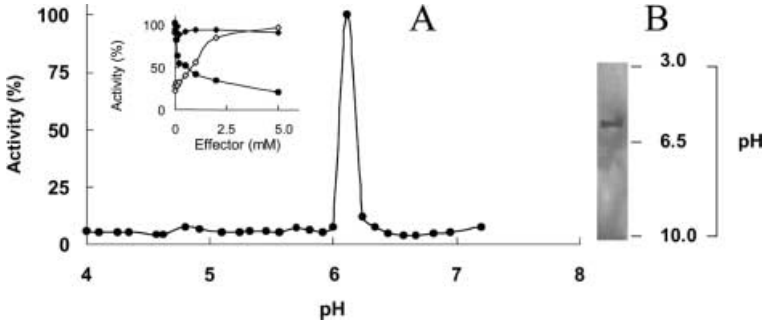

Fig. 5 Analysis of AGPase in crude extracts from wheat endosperm by chromatofocusing (**a**) and isoelectric focusing (**b**). **a** The sample was chromatofocused in a Mono P column (FPLC; Pharmacia, Sweden) using a pH gradient range 4–7: the column was equilibrated with 25 mM bis-Tris buffer adjusted to pH 7.1 with iminoacetic acid and eluted with Polybuffer 74-iminoacetic-acid (pH 4.0). Fractions eluted from the column were assayed for AGPase activity in the pyrophosphorolysis direction (assay A). **b** The same sample was electrofocused in the pH range 3–10 (under native conditions) according to O'Farrell (1975), and then the gel was electroblotted onto nitrocellulose membranes and probed with IgG prepared against the purified spinach leaf AGPase. *Inset* AGPase eluted from the chromatofocusing column was assayed for activity in the ADP-Glc synthesis direction (assay B) at the specified concentrations of Pi (*closed circles*), or 3PGA (*diamonds*), or in the presence of 2 mM Pi and different concentrations of 3PGA (*open circles*). 100% activity corresponds to 0.86 μmol·(ml sample)$^{-1}$ min^{-1}

the regulation and intracellular localization of AGPase from reserve tissues are matters of controversy. The enzyme from certain storage tissues was found to be insensitive to regulation (Hylton and Smith 1992; Kleczkowski et al. 1993; Weber et al. 1995; Rudi et al. 1997; Doan et al. 1999); furthermore, it was proposed that an unregulated AGPase from cereal endosperm is located predominantly in the cytosol (Kleczkowski 1996; Thorbjørnsen et al. 1996).

In this paper we describe the purification and characterization of AGPases from wheat leaves and endosperm. We found appreciable differences in the stability and regulatory properties of the enzymes from the two tissues. We determined that the sensitivity of the wheat endosperm AGPase to 3PGA (and to Fru-6-P) becomes apparent only when other metabolites (Pi, ADP, Fru-1,6-bisP) that behave as inhibitors are present.

The enzyme from wheat leaves is a typical leaf AGPase (Morell et al. 1987): it is a heterotetramer composed of two types of subunit (51 and 54 kDa), and is mainly regulated by 3PGA and Pi, with AMP, Fru-6-P, Fru-1,6-bisP and PEP behaving as minor activators. The enzyme was stable throughout the purification steps, including heat treatment.

Purification of wheat endosperm AGPase required a particular protocol, PEG and ammonium sulfate fractionations being two key steps for enzyme stability. Endosperm AGPase exhibited a native molecular mass of 210 kDa and migrated in SDS-PAGE gels as a wide protein band, suggesting resolution into subunits of 52 and 53 kDa. The heterotetrameric structure is typical of plant AGPases (Iglesias et al. 1994) and similar properties were reported for the potato tuber enzyme (Okita et al. 1990). Moreover, the size of the large subunit agrees with that deduced by Ainsworth and co-workers (1995) after cloning of the gene.

Wheat endosperm AGPase was insensitive to activation by a number of metabolites, but 3PGA could relieve the inhibition caused by Pi, ADP and Fru-1,6-bisP. Fructose-6-phosphate also relieved this inhibition, but less effectively. The insensitivity of maize endosperm AGPase to regulators is caused by proteolysis (Plaxton and Preiss 1987). To avoid this, we used protease inhibitors during cell disruption and optimized the purification procedure to favor enzyme stability and integrity. Although the possibility of some proteolysis cannot be discounted, we purified an AGPase with distinctive (novel) regulatory properties, rather than an unregulated enzyme. Chromatofocusing and isoelectric focusing showed that this AGPase is the major isoform, representing more of 95% of the enzyme (exhibiting AGPase activity and immunoreactivity) in wheat endosperm extracts. The unambiguous comparison of these results (considering the existence of multiple AGPase transcripts in wheat endosperm) needs to wait for the final characterization of wheat endosperm AGPase to be done using heterologous expression of defined cDNAs.

Our results suggest that AGPase activity may be finely regulated in the endosperm, and that the ratio 3PGA/Pi may determine the physiological levels of activity. Our results are different from reports showing insensitivity of barley endosperm AGPase to 3PGA and Pi (Kleczkowski et al. 1993; Rudi et al. 1997). Of course, the barley enzyme might be different, but other factors may account for the differences: (i) the higher degree of purification of the enzyme we used; (ii) our use of the ADP-Glc synthesis assay; and (iii) the study by Rudi et al. (1997) was performed with a recombinant protein having a $(His)_6$-tag at the N-terminus of the small subunit, which may alter its properties. In fact, expression of a potato tuber AGPase in which the N-terminus of the small subunit is slightly modified renders an enzyme with altered sensitivity to regulators (Iglesias et al. 1993; Ballicora et al. 1995). Recently, Doan et al. (1999) expressed the barley endosperm AGPase, unmodified at the N-terminus, and found that the small subunit alone exhibits 3PGA-dependent activity, as occurs with the unaltered potato tuber enzyme (Ballicora et al. 1995).

The metabolic routes operating in endosperm tissues are not well known, thus limiting our understanding of the relevance of the regulatory properties of the wheat AGPase to storage-cell metabolism. However, the results presented here confirm the crucial role of this enzyme in starch metabolism in the endosperm, as highlighted by:

i. High levels of activity per gram of fresh tissue and high affinity of the enzyme towards Glc-1-P and ATP.
ii. The inhibitory effect of Pi and ADP clearly will translate into decreasing enzyme activity when ATP levels are low in the endosperm.
iii. The reversal of inhibition by Fru-6-P could be relevant, as entrance of carbon to the amyloplast seems to occur mainly through hexose-phosphate transport, thus indicating a key role for these metabolites in endosperm metabolism (ap Rees and Entwistle 1989).
iv. The effect of 3PGA may be important if it is confirmed that the Pi–triose phosphate translocator exists in the amyloplast envelope and is operative when there are high levels of carbon flux into the storage tissues (Sivak and Preiss 1998).

Finally, the physiological relevance of inhibition by Fru-1,6-bisP is more difficult to envision but wheat endosperm amyloplasts lack fructose-1,6-bisphosphatase (ap Rees and Entwistle 1989) and levels of this metabolite could therefore increase under certain conditions in the cell.

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