BIOCHEMICAL STUDIES ON PLANT ADP-GLUCOSE PYROPHOSPHORYLASE

REGULATORY PROPERTIES

By

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Abstract

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ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis in plants. Plant AGPases are composed of two distinct subunits, a catalytic small subunit (SS) and a non-catalytic large subunit (LS). Previously, we have identified and characterized an allosteric $LS_{P52L}$ mutant, which when co-expressed with wild type SS, formed an enzyme with down-regulating allosteric properties. To further investigate the structure-function relationships between the two subunits with regard to allosteric regulation, random chemical mutagenesis was performed to generate SS suppressors of $LS_{P52L}$. Several putative SS mutant suppressors were identified by their ability to restore glycogen accumulation when co-expressed with $LS_{P52L}$ mutant in *Escherichia coli glgC-* strain. Kinetic analysis of these second-site mutant SS enzymes indicated that they comprise two distinct classes based on the SS interaction with $LS_{P52L}$ or wild type LS. One class contained bona fide SS suppressors ($SS_{L46F}$ and $SS_{P112L}$), which reversed the down-regulatory properties of $LS_{P52L}$ but not to wild type LS. The other SS class contained allosteric mutants, $SS_{P308L}$ and $SS_{R350K}$, which generated up-regulated enzymes with wild type LS as well as $LS_{P52L}$. These results indicate that both

LS and SS have a regulatory function in controlling allosteric properties through enhancing subunit interactions. In addition to allosteric regulation, plant AGPases are redox regulated by reduction of an intermolecular disulfide bond (S-S) between two Cys12 residues of catalytic small subunits (SSs). In this study, we replaced the Cys 12 residues with Ala ($SS_{C12A}$) to remove the disulfide bond in both the potato tuber and *Arabidopsis* leaf AGPase SSs. $SS_{C12A}$s were co-expressed with the corresponding LSs and resulting enzymes were purified. Kinetic analysis of $SS_{C12A}$ containing enzymes indicated that they have high affinity to the activator 3-phosphoglycerate and substrate ATP in both reducing and oxidizing conditions. Thermal stability of these enzymes was also investigated using both kinetics and biochemical approaches. $SS_{C12A}$ containing enzymes were less stable than the wild type enzymes at high temperatures, based on the results from circular dichroism spectroscopy and enzyme activity. Our results suggested the S-S bond in the AGPase is important for not only enhancing enzyme activation through higher affinity to activator and substrate, but also enzyme stability to maintain ordered structure at high temperatures.

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# General introduction

**Introduction**

The increase in crop productivity and yield is an important issue not only in agriculture and industry, but also in academic areas, which are related to the study of plant genetics and biochemistry. Starch is a major carbohydrate source in nature and is one of the target nutrients for agriculture and the food industry. Starch acts as a store for large amounts of fixed carbon from photosynthesis and is one of the main energy sources for plants. As a result of its significance, various approaches have been undertaken to engineer starch for enhancing crop quality and yield (Tjaden et al., 1999, Loef et al., 1999 and Regiere et al., 2002). Most of these studies have focused on controlling ADP-glucose (substrate of starch synthase) level by manipulating the enzymes which are involved in starch biosynthesis (Stark et al., 1992, Giroux et al., 1996 and Regiere et al., 2002).

The starch biosynthetic pathway consists of three different steps. These steps and the enzymes which catalyze them are as follows.

1) Glucose -1- phosphate + ATP $\leftrightarrow$ ADP-glucose + pyrophosphate

   (ADP-glucose pyrophosphorylase)

2) ADP-glucose + Amylose (n) $\rightarrow$ Amylose (n+1) + ADP

   (Starch synthase)

3) Amylose ($\alpha$ 1-4 ) $\rightarrow$ Amylopectin ($\alpha$ 1-4 and $\alpha$ 1-6)
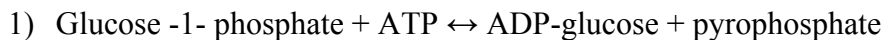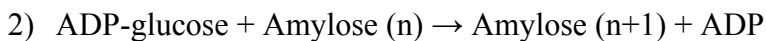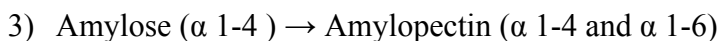
   (Branching enzyme)

The first step is the rate limiting reaction catalyzed by ADP-glucose pyrophosphorylase (AGPase). Therefore, AGPase has been extensively studied in order to increase its activity; enhancing both starch content and yield (Slattery et al., 2001 and Regiere et al., 2002).

**Structure of higher plant ADP-glucose pyrophosphorylase**

Higher plant AGPase has two subunit types which evolved from a common ancestral gene to form an active heterotetrameric ($\alpha_2 \beta_2$) enzyme (Okita et al., 1990). By contrast, the bacterial AGPase is encoded by a single gene which assembles to form a homotetrameric ($\alpha_4$) enzyme (Ballicora et al., 2003). Obvious questions that immediately come to mind is why the plant AGPases have two subunits rather than from the single type that makes the bacterial AGPases and what are the functions of each subunit type in terms of enzyme regulation and catalysis? Because of their size variability in different plant species and organs, the two subunits are called large subunit (LS) and small subunit (SS) although the molecular weight difference between the two subunits types is only 1 to 5 kilodaltons (kDa) (Ballicora et al., 2003).

There are several differences between the LS and SS. With reference to the potato tuber enzyme, the SS is a catalytic subunit, while the LS does not possess any enzyme activity by itself. The conservation of SS sequence is higher than that of LS among different species (Smith-White et al., 1992). An antibody against spinach AGPase SS recognizes SSs of other plants rather than the spinach AGPase LS (Okita et al., 1990). The LS has isoforms that are differentially expressed in each tissue within the plant,

while the single SS can form an active heterotetramer with different LSs regardless of its tissue specificity (Crevillen et al., 2005).

Recently, the crystal structure of inhibitor-bound potato tuber AGPase SS homotetramer was solved as well as the substrate (ATP and ADP glucose) bound forms (Jin et al., 2005). Each subunit consists of a catalytic N-terminus and a regulatory C-terminus which are connected by strong hydrophobic interactions and a long loop containing about twenty amino acids. This structure also depicted the intermolecular disulfide bond which connects a Cys residue of one SS to the equivalent Cys residue in the other SS. Three effector anion binding sites were identified in each subunit (total 12 sites in the tetramer), and this structure showed the nature of interaction between effector anions and amino acid residues involved in anion binding. Most of these amino acid residues involved in anion binding have been identified and characterized by biochemical and kinetic studies in terms of allosteric regulation except for the amino acid residues of site 3 (Jin et al., 2005). The location of the ATP binding sites was also shown in this structure (Jin et al., 2005). It is still unclear how the allosteric regulatory sites communicate with the active site. The exact mechanism of enzyme catalysis and regulation can not be fully understood until the activated heterotetramer crystal structure is available.

**Regulatory properties of plant ADP-glucose pyrophosphorylase**

Both bacterial and plant AGPases are regulated by small effector molecules which are intermediates of the major carbon utilization processes in these organism (Ballicora et al., 2003 and 2004). In photosynthetic tissues of higher plants, most AGPases are

activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi) (Ballicora et al., 2004). It has been suggested that the ratio of 3-PGA and Pi is important for the regulation of the AGPase during the light and dark cycle (Preiss et al., 1989). Unlike the leaf AGPases, the activation by 3-PGA varies in sink tissues. Wheat and barley endosperm AGPases show little response to 3-PGA (Duffus et al., 1992; Kleczkowski et al., 1993). However, maize endosperm and developing rice seed AGPases show a substantial increase in enzyme activity in the presence of 3-PGA (Krapp et al., 1995; Sikka et al., 2001) although stimulation of maize enzyme activity varies depending on the study (Plaxton et al., 1987; Hannah et al., 1995; Cross et al., 2004). Recent studies showed that starch synthesis increases without a change in 3-PGA level when leaf sugar levels are increased, and enhanced 3-PGA concentration did not affect starch accumulation in leaves (Krapp et al., 1995; Geigenberger et al., 1996; Westram et al., 2002). These studies suggested that additional factors may be involved in controlling starch synthesis in leaf tissue.

In addition to the allosteric regulation by small effector molecules, the higher plant AGPases are redox regulated by reversible disulfide bond formation (Geigenberger et al., 2005). The higher plant AGPases have one intermolecular disulfide bond between the two SSs except for the monocot endosperm AGPases which do not have this conserved SS Cys that makes the intersubunit disulfide bond (Ballicora et al., 1999). In photosynthetic tissues, light activated thioredoxin, through ferredoxin-thioredoxin reductase, reduces the disulfide bond that, in turn, activates the AGPase. Oxidized thioredoxin can inactivate the AGPase by the formation of a disulfide bond (Buchanan et al., 2005). In sink organs, there is no photosynthetic electron transport which can

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generate reducing power to activate thioredoxin. Therefore, ferredoxin is reduced by NADPH via ferredoxin-NADP reductase in amyloplasts, and the reduced ferredoxin can activate thioredoxin.

Recently, the Buchanan group identified a set of thioredoxin-interacting proteins in amyloplasts isolated from wheat endosperm by using affinity column chromatography (Balmer et al., 2006). Most of affinity-purified proteins were previously identified in chloroplasts isolated from various plants (Motohashi et al., 2001; Marchand et al., 2004). AGPase LS and SS were also identified as binding partners of thioredoxin in both chloroplasts and amyloplasts. These biochemical results support the view that the redox regulation is effective in both photosynthetic and nonphotosynthetic tissues. Moreover, they also suggest that thioredoxin has a general role in metabolite regulation through reducing power in both sink and source tissues. However, the biological and physiological relevance of this thioredoxin mediated mechanism has not been demonstrated in most cases.

The redox-activation of AGPase is also triggered by sugars through an unknown mechanism (Geigenberger et al., 2005). It has been reported that the addition of sugars activated the potato tuber and Arabidopsis leaf AGPases (Tiessen et al., 2002; Hendricks et al., 2003; Kolbe et al., 2005; Lunn et al., 2006). These studies showed that the supply of sugars leads to stimulation of starch synthesis without increasing the metabolite level. The detailed mechanisms for sugar induced activation of AGPase need further investigation.

**Structure and functional relationship of plant ADP-glucose pyrophosphorylase**

Although the potato tuber AGPase SS can form an active homotetrameric enzyme with low sensitivity to the activator 3-PGA, the LS is unable to make an active enzyme by itself due to its low solubility. When the LS and SS assemble to form a heterotetrameric enzyme, this enzyme is highly sensitive to the activator 3-PGA.

The functions of the two subunit types have been demonstrated by numerous studies using site-directed mutagenesis (Hill et al., 1991; Fu et al., 1998; Ballicora et al., 1998). The conserved residues among bacteria and plant AGPases, which are important for substrate binding, were selected, and the kinetic parameters of these mutant enzymes were studied. The equivalent residues in the potato tuber AGPase LS and SS for glucose-1-phosphate binding (Lys 195 in *E. coli*) and metal binding (Asp 142 in *E. coli*) were selected for site directed mutagenesis (Hill et al., 1991; Fu et al., 1998). The studies of these mutant enzymes indicated that mutations in the SS have more drastic effects on catalytic activity than those in the LS. The SS mutant enzymes showed decreased apparent affinity of substrates, but the LS mutant enzyme did not. Therefore, it is believed that the SS has a catalytic role and the LS has a regulatory role. This functional assignment has been also supported by genetic studies using random mutagenesis. Several allosteric LS mutants were identified as either up-regulatory (E38K and P66L) or down-regulatory mutants (P52L) in potato tuber AGPase LS (Greene et al., 1996; 1998).

The effector binding sites in spinach leaf AGPase LS and SS were identified by labeling with pyridoxal phosphate which is a 3-PGA analog (Ball et al., 1994). Only a single reactive site was observed for the SS. By contrast, three sites were phosphopyridoxylated in the spinach leaf LS. Site directed mutagenesis of these sites in potato tuber AGPase indicated that the affinity of 3-PGA was significantly lower in the

SS effector binding sites than those of the LS (Ballicora et al., 1998). The substrate binding and regulatory potato tuber SS mutants were also identified by random mutagenesis (Laughlin et al., 1998; Salamone et al., 2000; 2002). These results suggested that the SS has not only catalytic but also a regulatory role and that the LS is not a regulatory subunit but simply modulates the regulatory properties of SS. This classical functional assignment for each subunit type has been challenged by results from other groups. It has been reported that synergistic interactions between LS and SS determine the regulatory properties of potato tuber AGPase (Hwang et al., 2005). The kinetic results of the potato and maize mosaic enzymes suggested that both subunit types contribute to the allosteric regulatory properties (Cross et al., 2004). The results from random mutagenesis of maize AGPases indicated that both subunits are equally sensitive to mutations which affect enzyme activity when they are expressed in bacteria (Georgelis et al., 2007). Our recent results support a role for both subunit types in not only allosteric regulation, but also enzyme catalysis through enhancing subunit interaction (Hwang et al., 2006; 2007).

The N-terminal region of SS is known to be critical for enzyme catalysis since this is where the active site is located. The catalytic implications of LS N-terminal regions were investigated by a leucine scanning mutagenesis of the six N-terminal proline residues observed in the first 66 residues (Hwang et al., 2007). Three Pro residues were found to be important for enzyme regulation and catalysis. These results suggested that the N-terminal region of LS is responsible for not only allosteric regulation, but also enzyme catalysis. In addition to the characterization of LS proline mutants, we also showed that the LS can be labeled by 8-azido-ATP (an analog of the substrate ATP), as

efficiently as the SS (Hwang et al., 2006; 2007).  In these labeling and kinetic studies of the LS, the LS has  catalytic potential and can bind substrates like the SS, which, in turn, indirectly affects  enzyme catalysis (Hwang et al., 2006; 2007).  Photoaffinity labeling studies enabled the identification of the LS ATP binding site (Hwang et al., 2006).  Mutation of putative LS ATP binding sites caused changes in the apparent affinity for the substrates (ATP and glucose-1-phoshate) as well as the activator (3-PGA).  The study of the LS ATP binding site also supported the hypothesis that ATP binds to LS and the LS can affect enzyme catalysis as well as allosteric regulation (Hwang et al., 2006).  These results strengthened the conclusion that the LS can actively participate in catalysis by enhancing the apparent affinity for activator and substrate.  However, the limitation of this speculation is that these results were obtained from heterotetrameric enzymes.  The study of the LS homotetrameric enzyme is required.

**The nature of LS: SS dependent or independent subunit?**

Potato, tomato and *Arabidopsis* have multiple LS isoforms, but only contain a single SS (La Cognate et al., 1995; Park et al., 1998; Crevillion et al., 2003).  The single *Arabidopsis* SS gene is expressed in all tissues, while the different *Arabidopsis* LSs are expressed in a tissue specific manner (Crevillion et al., 2005).  The divergence and tissue specificity of LSs suggested that this subunit determines the AGPase activity in a spatial and temporal manner.

These different isoforms of *Arabidopsis* LS have been characterized in terms of both their kinetic and regulatory properties (Crevillion et al., 2003).  In *Arabidopsis*, two SS genes (ApS1 and ApS2) and four LS genes (ApL1, ApL2, ApL3, and ApL4) were

identified by genomic database searches. ApS2 is a pseudo gene with no catalytic activity (Crevillion et al., 2003). These *Arabidopsis* LS isoforms conferred different kinetic and regulatory properties when they assembled with SS as a heterotetramer (Crevillion et al., 2003). In addition to biochemical results at the enzyme level, the tissue specific expression pattern and sugar mediated regulation of these *Arabidopsis* LS isoforms has also been reported (Crevillion et al., 2005). There is a correlation between enzyme regulatory properties and tissue dependent expression pattern. The ApL1 is a leaf type LS which shows higher sensitivity to 3-PGA and is predominantly expressed in leaf tissue. The other LS isoforms are sink type LSs which are less dependent on the 3-PGA/Pi ratio and can be regulated by sugars. Interestingly, these heterotetramer enzymes of different LS isoforms showed distinct affinity for activator as well as substrates (Crevillion et al., 2005). These results indicate that the LS is not only responsible for the distinct allosteric regulatory properties, but also for the affinity of substrates of the various AGPase forms in different tissues.

Recently, we identified a potato tuber AGPase LS mutant which has enhanced LS solubility and the capacity to form a soluble LS homotetramer. This LS homotetramer has very little enzyme activity and is not responsive to the activator 3-PGA. However, the LS mutants showed enhanced enzyme activity and regulation by 3-PGA when assembled with the catalytically defective SS. This result indicated that the solubility of LS can be increased by a single mutation (Ser302 to Asn), and catalytic and regulatory properties are restored when assembled with the SS. It has been demonstrated that the catalytic activity of potato tuber AGPase is also enhanced by two mutations in LS (Ballicora et al., 2005). In addition to the LS, the sensitivity to activator of the SS

homotetramer was increased to a level greater than that of the wild type heterotetramer enzyme by several point mutations (Salamone et al., 2000; 2002). Taking all of these results together, the roles of LS and SS can be restored or modified by mutations. This is supported by the fact that both subunit types evolved from a single ancestor, are equally important in enzyme catalysis and regulation, and are interdependent to each other.

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# Chapter two

# Subunit interactions specify the allosteric properties of

# potato tuber ADP-glucose pyrophosphorylase

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**Abstract**

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis in plants. The potato tuber enzyme contains a pair of catalytic small subunit (SS) and a pair of non-catalytic large subunit (LS). We have previously identified a LS mutant containing a $P_{52}L$ replacement, which rendered the enzyme with down-regulatory properties. To investigate the structure-function relationships between the two subunits with regard to allosteric regulation, putative SS mutants that could reverse the down-regulatory condition of $LS_{P52L}$ were identified by their ability to restore glycogen accumulation in an AGPase-deficient *Escherichia coli glgC-* strain. Two distinct LS-dependent classes, bona fide SS suppressors dependent on $LS_{P52L}$ but not $LS_{WT}$ and SS up-regulating allosteric mutants, were evident by kinetic analysis. These results indicate that both LS and SS have a regulatory function in controlling allosteric properties through enhancing cooperative subunit interactions.

**Introduction**

Synthesis of starch and glycogen initiates with the formation of ADP-glucose catalyzed by ADP-glucose pyrophosphorylase (AGPase). Both plant and bacterial AGPases are allosterically regulated by small effector molecules [1]. The plant AGPase is activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic orthophosphate (Pi). Unlike the bacterial enzyme, which consists of four identical subunits, the higher plant AGPase is composed of two distinct subunits, large (LS) and small subunit (SS) to form a heterotetramer ($L_2S_2$) structure. The two subunit types have different roles in enzyme function. The potato tuber AGPase SS is capable of forming a catalytically active homotetramer but with defective allosteric regulatory properties, whereas the LS is unable to form an active enzyme [2]. Although both subunits of the potato tuber enzyme interact with pyridoxal phosphate, which mimics the activator 3-PGA [3], site-directed mutagenesis studies of residues suspected in activator binding showed that only those in the SS have a drastic effect on the enzyme's allosteric regulatory properties [4]. These results suggested that the LS is not a regulatory subunit but simply modulates the allosteric regulatory properties. Other studies [5-8] have supported a role for LS in both catalysis and allosteric regulation. Allosteric regulatory properties of mosaic enzymes containing maize endosperm and potato tuber AGPase SSs and LSs suggested that both LS and SS are regulatory subunits [7]. Based on the analysis of various combinations of mutant potato tuber AGPase LSs and SSs, the regulatory properties of AGPase have been demonstrated to be synergistic and not simply an additive product of the regulatory properties of the LS and SS [8]. Recently, we reported that the potato tuber AGPase LS can bind ATP as efficient as the SS, and the LS can contribute to catalysis indirectly as

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well as allosteric regulation [9]. This result indicated that the LS is important not only in specifying the allosteric regulatory properties of the heterotetrameric enzyme but also in substrate binding and net catalytic activity of the oligomeric enzyme.

We had previously identified a potato tuber AGPase LS containing a P52L replacement ($LS_{P52L}$), which when combined with wild type SS formed a down-regulatory enzyme, *i.e.* less sensitive to activation by 3-PGA [10]. In this study, we undertook a biochemical genetic approach to gain further insight on the roles of the LS and SS in specifying the allosteric regulatory properties of the heterotetrameric enzyme. Wild type SS cDNA was subject to random mutagenesis and then co-expressed with $LS_{P52L}$ whereupon the cells were screened for their capacity to restore glycogen production in *Escherichia coli (E. coli) glgC-* strain. Seven cell lines were identified which showed elevated levels of glycogen accumulation than cells expressing an enzyme composed of wild type SS ($SS_{WT}$) and $LS_{P52L}$. The variant enzymes from four cell lines were purified and analyzed for their allosteric and kinetic properties. Two distinct types of SS mutants were evident: SS up-regulatory allosteric mutants that were independent of the LS type it was assembled with or bona fide suppressors of $LS_{P52L}$. The results show that the loss-of-function imposed by $LS_{P52L}$ can be reversed by specific mutations in the SS and that the allosteric regulatory properties are contributed by both subunit types.

## Materials and Methods

### Chemical mutagenesis

Mutagenesis was performed on pSH228, a bacterial expression plasmid of the potato tuber AGPase SS cDNA, in the presence of 0.8 M hydroxylamine, 50 mM sodium

phosphate pH 6.0 and 1 mM EDTA at 37°C for 30 hours. The mutant DNAs were transformed into *E. coli* AC70R1-504 which lack endogenous AGPase and co-expressed with the $LS_{P52L}$ expression plasmid pTG16. After overnight growth, the colonies were screened for their ability to restore glycogen production as assessed by iodine staining [10]. The SS DNAs from strongly iodine staining clones were obtained and recloned into pSH208 which is a backbone plasmid of pSH228 for their expression and purification [11].

**Expression and purification of wild type and mutants enzymes**

Wild type and mutants SS DNAs were co-expressed with wild type LS or $LS_{P52L}$ DNA in EA345 cell line. Enzyme purification methods were as described [11] except that the final hydrophobic interaction column purification step was replaced by POROS 20HQ chromatography. The nearly pure enzyme was loaded onto the HQ column, washed with 0.15 M NaCl and the enzyme was eluted with 0.3 M NaCl. The pure 0.3 M NaCl elution enzyme fractions were collected and diluted with 2 volumes of HQ column binding buffer (25 mM HEPES-NaOH, pH 8.0, and 5 % glycerol). The enzyme solution was concentrated by ultrafiltration (cut-off MW 10 kDa; Milipore), aliquoted and stored at -80 °C freezer until analyzed. The purity of the enzyme at each chromatography step was assessed by SDS-PAGE.

**Modeling structure**

The three dimensional structure of the SS monomer was modeled based on the potato SS homotetramer structure (Jin et al., 2005) (Figure 1). DeepView, the Swiss-PdbViewer (http://www.expasy.org/spdbv/), and POV-Ray, the ray-tracing software,

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([http://www.povray.org/](http://www.povray.org/)) were used for modeling the structure. The protein structure of the potato small subunit homotetramer was illustrated using Ribbons (ver. 3.32) [12].

**ADP glucose synthesis assay**

The reaction was performed at 37 °C in 0.1 ml of 100 mM HEPES-NaOH, pH 7.0, 7 mM MgCl$_2$, 3 mM DTT, 5 mM 3-PGA, 0.4 mg/ml bovine serum albumin (BSA), 0.15 U inorganic pyrophosphatase (Sigma), 1.5 mM ATP, 1.5 mM Glc-1-P, and [$^{14}$C] Glc-1-P (1000-1200 dpm/nmol) as described in [13].

**Results**

**Isolation and identification of putative SS suppressors**

Chemical mutagenesis by hydroxylamine is an effective method for generating point mutations in DNA and has been effectively employed to generate regulatory and catalytic AGPase mutants [10, 14]. One point mutation in the LS resulted in a P52L replacement which rendered the resulting heterotetrameric enzyme with down regulatory properties, *i.e.* required higher levels of the activator 3-PGA for maximal activity and was more sensitive to inhibition by Pi [10]. This result supported a regulatory role for the LS, although, based on the effect of mutations at the putative effector binding sites of the LS and SS, it was concluded that the LS is not a regulatory subunit but simply modulates the regulatory properties of the SS [4]. To gain more insight on the subunit interactions specifying allosteric regulation, we attempted to identify SS suppressors which could reverse the down-regulatory phenotype mediated by LS$_{P52L}$.

To generate mutations, AGPase SS cDNA was treated with hydroxylamine as described in Materials and Methods, and then co-transformed with LS$_{P52L}$ in a glycogen-deficient *E. coli* strain lacking the AGPase structural gene *glgC*. Putative SS suppressors,

which would regenerate an active heterotetrameric enzyme, were initially identified by the restoration of glycogen production. Upon screening more than 10,000 bacterial colonies for glycogen accumulation by iodine staining, seven putative SS suppressors were identified. Four mutant lines showed a wild type level of iodine staining while three mutants showed much weaker signals (Table 1). DNA sequence analysis of the four SS cDNAs isolated from the strong iodine staining mutant lines showed that the mutations fell into two classes. One class consisted of $SS_{L46F}$ and $SS_{P112L}$, which are located close to the ATP binding site [15]. The other class, $SS_{P308L}$ and $SS_{R350K}$, are located in the C-terminal region which is adjacent to the putative 3-PGA binding sites (Figure 1).

**Purification and enzymatic characterization of SS mutant enzymes containing $LS_{P52L}$ and wild type LS**

The four SS mutants which resulted in wild type level of glycogen accumulation were selected for further characterization. These SS mutants were co-expressed with $LS_{P52L}$ or wild type LS ($LS_{WT}$); purified to near homogeneity and their kinetic parameters were analyzed using ADP-glucose synthesis assay (Table 2). In nearly all instances, the apparent affinities for ATP and glucose-1-phosphate by the double mutant enzymes were similar to those of the parent $LS_{P52L}/SS_{WT}$. The one exception was $LS_{P52L}/SS_{P112L}$, which showed a two-fold lower $S_{0.5}$ for glucose-1-phosphate than that seen for $LS_{P52L}/SS_{WT}$. By contrast, allosteric regulatory properties varied considerably among the four double mutant enzymes. $LS_{P52L}/SS_{L46F}$ and $LS_{P52L}/SS_{P112L}$ showed only moderate increases (2- to 3-fold) in the affinity for 3-PGA (activator) and 0- to 2-fold increases in resistance to Pi inhibition than $LS_{P52L}/SS_{WT}$. Their $A_{0.5}$ for 3-PGA and $I_{0.5}$ for Pi were correspondingly

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higher and lower than the values measured for the $LS_{WT}/SS_{WT}$. The other two enzymes, $LS_{P52L}/SS_{P308L}$ and $LS_{P52L}/SS_{R350K}$, showed even greater sensitivity to 3-PGA activation (4- to 7-fold) and resistance to Pi inhibition (2- to 3-fold) than wild type enzyme.

To investigate whether these SS mutants were actually suppressors of $LS_{P52L}$ or LS independent, the four mutant SSs were co-expressed with wild type LS and the kinetic properties of the purified enzymes were studied (Table 3). The apparent substrate binding affinity values of the four enzymes were similar to those of wild type enzyme (less than 2-fold). The responses to 3-PGA and Pi, however, showed two different patterns. The $A_{0.5}$ values of $LS_{WT}/SS_{L46F}$ and $LS_{WT}/SS_{P112L}$ were nearly the same as wild type enzyme, indicating that the enhancement in 3-PGA activation seen with $LS_{P52L}$ is not evident with wild type LS. By contrast, the $A_{0.5}$ values of $LS_{WT}/SS_{P308L}$ and $LS_{WT}/SS_{R350K}$ were 10-fold lower than the wild type enzyme. Therefore, $SS_{L46F}$ and $SS_{P112L}$, are bona fide suppressors of $LS_{P52L}$ with regard to 3-PGA activation while $SS_{P308L}$ and $SS_{R350K}$ are LS-independent, SS up-regulated allosteric mutants. Unlike the distinct patterns seen for 3-PGA activation responses, most of $LS_{WT}/SS_{mutant}$ enzymes showed increased resistance to Pi inhibition than the wild type enzyme except $LS_{WT}/SS_{L46F}$.

**Discussion**

**Identification and characterization of novel classes of allosteric mutants**

Here we report the identification and characterization of two novel classes of SS allosteric mutants in their capacity to reverse the down-regulatory properties of the heterotetrameric enzyme imposed by the mutant $LS_{P52L}$. One class, consisting of $SS_{L46F}$

and $SS_{P112L'}$ are $LS_{P52L}$ suppressors as they increase the enzyme's sensitivity to 3-PGA only when assembled with this mutant LS type but not with wild type LS. The other class, consisting of $SS_{P308L}$ and $SS_{R350K}$, are LS type-independent as they increase the sensitivity of the resulting heterotetrameric enzyme to 3-PGA activation and resistance to Pi inhibition irrespective of the LS type with stronger responses seen with $LS_{WT}$ than with $LS_{P52L}$. The extent of up-regulation of the resulting heterotetrameric enzymes formed by these SS mutants was significantly higher when assembled with $LS_{WT}$ than $LS_{P52L}$, consistent with their synergistic interaction. The up-regulatory properties conferred by $SS_{P308L}$ and $SS_{R350K}$ are absolutely dependent on the presence of a LS, as homotetrameric enzymes composed of either $SS_{P308L}$ and $SS_{R350K}$ remain defective in allosteric regulatory properties and bacteria cells expressing these enzyme forms failed to accumulate glycogen (results not shown). Hence, the effect of these SS mutations is only evident in the context of the presence of the LS, further evidence for the allosteric regulatory properties of the heterotetrameric enzyme being specified by LS-SS interactions.

**Modeling analysis of SS suppressor mutants supports subunit interactions determining allosteric properties**

Recently, the structure of the potato tuber AGPase SS homotetramer ($S_4$) has been reported [15]. The $S_4$ structure is similar to the heterotetrameric enzyme ($L_2S_2$) in consisting of two A-type SSs linked by an interchain disulfide bond and two B-type SSs, the latter comparable to LSs in lacking an interchain disulfide bond. Because the potato tuber AGPase LS and SS sequences share about 55% identity with residues located in the

effector binding and substrate binding sites conserved in both the LS and SS, the structure of the heterotetrameric enzyme can be modeled where B type SS sequences are replaced by LS sequences (Figure 2). Such modeling shows that the N- and C-terminal domains of the LS lie adjacent to the corresponding domains in the SS in a head to head and tail to tail configuration. The N-terminal domain in both subunits is especially important in enzyme function as this region contains the substrate binding sites as well as residues which participate in effector anion binding. Each subunit of the modeled heterotetrameric enzyme contains three effector anion sites with one site composed of residues from both subunits based on the homotetramer structure. In the SS N-terminal region, residues that participate in effector anion binding include R39 and R51 for site 1, R51, R81 and H82 for site 2, and K67, H132 and T133 for site 3. Site 3 also contains R91 of the LS (equivalent to R81 of the SS). Both subunits have the highly conserved PAVP motif located at residues 52-55 in the LS and residues 42-45 in the SS which are important for ATP binding and allosteric regulation [10].

Analysis of the modeled heterotetrameric enzyme structure shows that all four mutations in the SS that give rise to increase 3-PGA activation are not located close to the $P_{52}L$ mutation in the LS (Figure 2). Hence, the increase in the enzyme's response to 3-PGA activation by these mutations is not due to a direct interaction between the mutated residues in the SS with the LS $P_{52}L$ but likely a result of a long distance interaction. Interestingly, the down-regulatory properties imposed by the $P_{52}L$ mutation in the LS $P_{52}AVP$ peptide are reversed by mutation at L46 located adjacent to this conserved $P_{42}AVP$ sequence motif in the SS. As the regulatory properties of the heterotetrameric enzyme are an interactive product of LS-SS synergy, the simplest explanation that

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accounts for this suppression effect is that the conformation changes at the LS N-terminal region mediated by P52L may be compensated by the N-terminal conformational changes induced by L46F mutation in the SS.

Similar subtle compensatory conformational changes in $SS_{P112L}$ when assembled with $LS_{P52L}$ are likely responsible for observed restoration of normal allosteric regulatory properties of the resulting heterotetrameric enzyme as well as the increases its apparent affinity for the substrate Glc 1-P. The $P_{112}$ residue is located in a loop region (residues 103 to 115) which moves significantly to accommodate binding of ATP [15]. Although the $P_{112}$L mutation does not alter the apparent affinity for ATP, it facilitates subsequent entry and binding of the hexose phosphate. Much more difficult to explain is the observed increase in 3-PGA activation by $SS_{P112L}$. The $P_{112}$L mutation is located in close proximity to anion site 3 which is made up of residues from both the LS and SS (R91 of the LS [equivalent to R81 of the SS] and K67, H132 and T133 of the SS [15]). Hence, the subtle structural changes imposed by P52L in the LS may be complemented by a structural change of the altered 103-115 loop which restores normal allosteric regulatory properties of the heterotetrameric enzyme.

Like the N-terminal mutations, the two C-terminal mutations ($SS_{P308L}$ and $SS_{R350K}$) that confer a LS-type independent activation response are located far from the LS $P_{52}$L. Both mutations, however, are located close to effector binding sites (Figure 1). The P308 residue lies in a loop (residues 298 to 318) which connects the N- and C-terminal domains within a subunit and contains Q312 and R314 which comprise part of effector anion site 2 [15]. The R350 residue is located in the C-terminal domain and lies within the same pocket with K402 and K439 which constitute part of effector anion site 1

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[15] (Figure 1). The apparent absence of LS-type specificity by these C-terminal mutations in the SS suggests that they may increase the affinity of the SS for the activator 3-PGA directly. However, since the up-regulatory effects mediated by these SS mutations are only evident in the heterotetrameric and not homotetrameric enzyme, it is more likely that the subtle structural changes mediated by the SS C-terminal mutations are transmitted through its interactions with the LS C-terminal domain to increase the enzyme's affinity towards 3-PGA. In support of subunit interactions, Cross et al. [16] has shown that a specific 55 amino acid peptide (296 to 350) of the potato tuber SS is sufficient to permit a functional assembly of the maize SS with the potato tuber LS and confer normal catalytic and allosteric regulatory properties to the potato-maize mosaic enzyme . Both LS-type independent mutant residues, P308L and R350K, are located in this peptide. Our results provide additional evidence that this 55 amino acid motif is important for subunit interaction in terms of allosteric regulation.

**Both LS and SS are equally important in enzyme catalysis and regulation**

Results from site-directed mutagenesis studies of residues suggested to participate in catalysis and allosteric regulation indicate that the two subunit types play different roles in enzyme catalysis and regulation [4 and 17]. The SS has both catalytic and regulatory properties, while the LS modulates the regulatory properties of the SS [1]. This suggested functional assignment for each subunit type, however, is not entirely consistent with other studies [7, 9, 11 and 16] where the catalytic and regulatory properties of the enzyme were affected by mutations in both LSs and SSs of maize and potato tuber AGPases. Moreover, random mutagenesis of the maize AGPase SS and LS indicated that both subunits are equally sensitive to mutations which affect enzyme

activity when they are co-expressed in bacteria [18]. These results together with those presented here indicate that both subunits contributed not only to regulatory properties, but also for net enzyme catalysis.

Interestingly, of the four SS mutations identified in this study, only P308 and R350 are conserved in the LS. Kinetic results of purified enzymes containing $LS_{P52L}$ $_{R371K}/SS_{WT}$ or $LS_{P52L}$ $_{P329L}/SS_{WT}$ indicated that the enzymes showed similar down-regulatory properties as $LS_{P52L}/SS_{WT}$ enzyme (result is not shown). Hence, the effect of these mutations may be only effective in the SS and when this subunit is assembled with the LS. This observation provides additional support for the role of LS-SS interactions in specifying the allosteric regulatory properties of the heterotetrameric enzyme.

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**Figure legends**

**Figure 1.    Location of the mutations in the crystal structure of the potato tuber AGPase SS monomer**

The structure of the potato tuber AGPase SS monomer was modeled based on the recently published structure of the potato tuber SS homotetramer [14].    Residues that participate in effector anion binding include K402 and K439 for site 1, R314 and Q312 for site 2, and site 3 composed of residues from both subunits (not shown here).    The four SS mutations (L46, P112, P308 and R350) identified in this study are located near anion effector binding sites.

**Figure 2. Modeled heterotetrameric structure of the potato tuber AGPase**

A and A' indicate small subunits (SS), and B and B' indicate large subunit (LS). The four SS mutations are shown in A and A', and P52L is shown in B and B'.
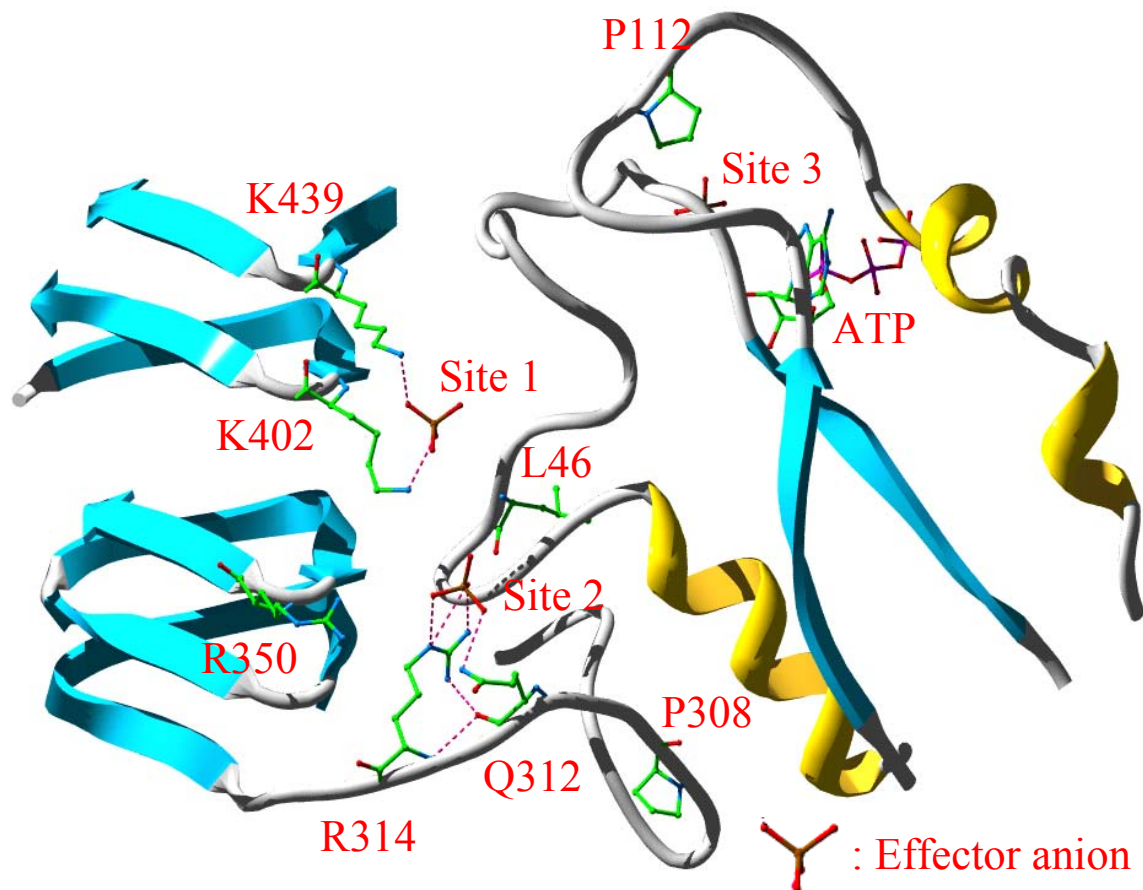
**Figure 1.**

**Figure 2.**
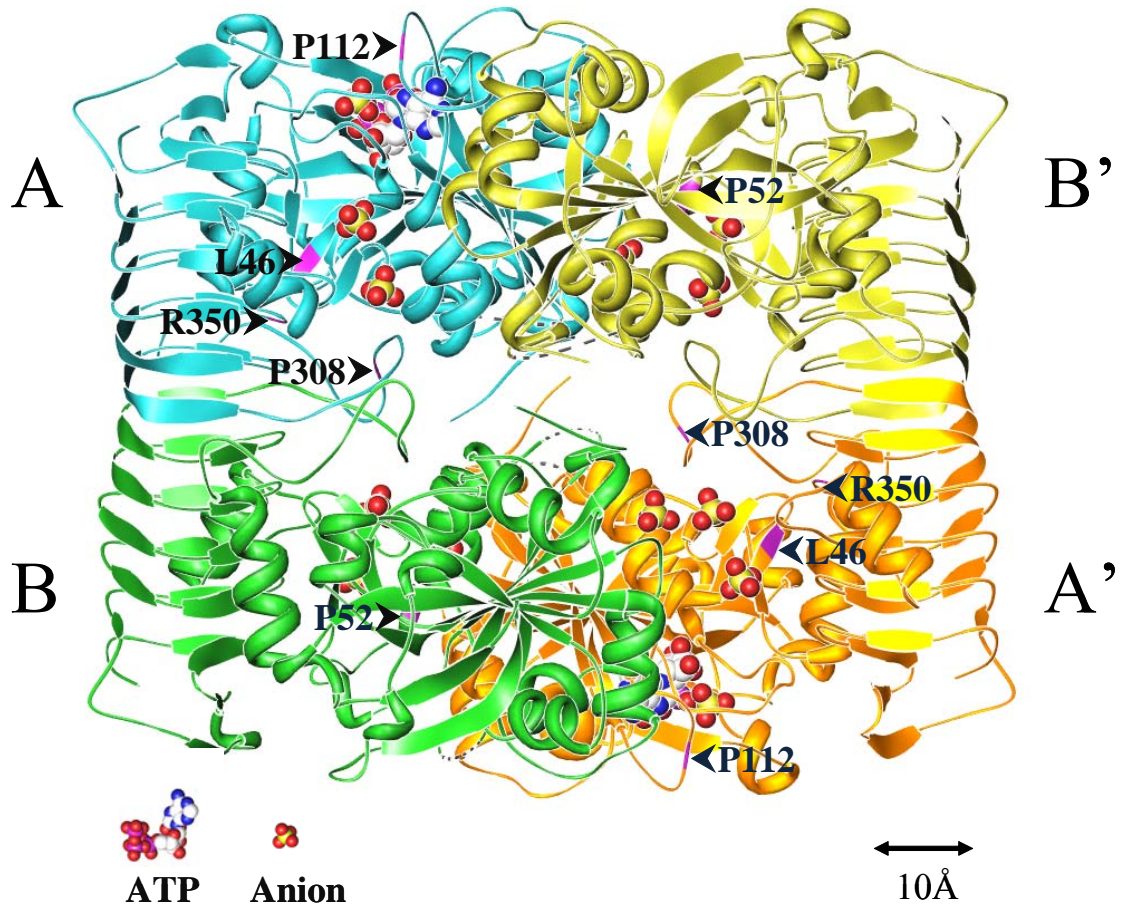
**Table 1. Identification of putative SS suppressors by iodine staining of bacterial cells expressing LS$_{P52L}$ and mutant SS.**

Seven putative SS suppressors were identified based on their capacity to restore glycogen accumulation when co-expressed with the down-regulatory LS$_{P52L}$.   The original residues, their position and replacement in the SS are shown.  Cells expressing wild type enzyme have an iodine staining level of +++ while cells expressing wild type SS and LS$_{P52L}$ do not stain with iodine.

| wild type residues | Position | Replacement residues | Iodine staining level |
|---|---|---|---|
| Leu | 46 | Phe | +++ |
| Pro | 112 | Leu | +++ |
| Pro | 308 | Leu | +++ |
| Arg | 350 | Lys | +++ |
| Pro | 45 | Leu | ++ |
| Val/Pro | 44/45 | Ala/Leu | + |
| Arg | 51 | Leu | + |

**Table 2. Kinetic parameters of Enzymes containing SS mutants when assembled with LS$_{P52L}$**

Kinetic values were obtained by assaying the various enzymes using the ADP-glucose synthesis direction. The mean values (in mM) have a standard error of less than 15% in all cases. WT denotes wild type. The apparent $A_{0.5,}$ and $S_{0.5}$ for 3-PGA and substrates, respectively, correspond to the concentration of these molecules required for the enzyme activity to attain 50% of maximum activity. The $I_{0.5}$ is the amount of Pi required to inhibit the enzyme activity by 50% of maximal activity.

| LS | WT | P52L | P52L | P52L | P52L | P52L |
|---|---|---|---|---|---|---|
| SS | WT | WT | L46F | P112L | P308L | R350K |
| $A_{0.5}$ (mM) for 3-PGA | 0.04 | 0.27 | 0.1 | 0.14 | 0.006 | 0.01 |
| $I_{0.5}$ (mM) @ | | | | | | |
| 0.5 mM 3-PGA | 0.54 | 0.52 | 0.61 | 0.43 | 2.5 | 1.9 |
| 5 mM 3-PGA | 3.5 | 1.2 | 1 | 2.3 | 6.2 | 9 |
| $S_{0.5}$ (mM) | | | | | | |
| ATP | 0.17 | 0.25 | 0.24 | 0.22 | 0.24 | 0.29 |
| Glc-1-P | 0.13 | 0.42 | 0.36 | 0.21 | 0.39 | 0.41 |

**Table 3. Kinetic parameters of SS mutants when assembled with wild type LS.**

Kinetic parameters of wild type and LS$_{P52L}$ enzyme were taken from table 2. Kinetic values were obtained by assaying the various enzymes using the ADP-glucose synthesis direction. The mean values (in mM) have a standard error of less than 15% in all cases. The definition of $A_{0.5}$, $I_{0.5}$, and $S_{0.5}$ is the same as the Table 2.

| LS | WT | P52L | WT | WT | WT | WT |
|---|---|---|---|---|---|---|
| SS | WT | WT | L46F | P112L | P308L | R350K |
| $A_{0.5}$ (mM) for 3-PGA | 0.04 | 0.27 | 0.05 | 0.03 | 0.003 | 0.004 |
| $I_{0.5}$ (mM) @ | | | | | | |
| 5 mM 3-PGA | 3.5 | 1.2 | 2.5 | 7 | 12.1 | 9.6 |
| $S_{0.5}$ (mM) | | | | | | |
| ATP | 0.17 | 0.25 | 0.17 | 0.11 | 0.21 | 0.11 |
| Glc-1-P | 0.13 | 0.42 | 0.26 | 0.21 | 0.21 | 0.2 |

# Chapter three

# Disulfide bond is critical for the enzyme regulation and heat stability of plant ADP-glucose pyrophosphorylase

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**Abstract**

The potato tuber ADP-glucose pyrophosphorylase (AGPase), which catalyzes the first committed step of starch biosynthesis, is regulated by redox potential where the reduction of an intermolecular disulfide bond (S-S bond) between two Cys-12 residues of the catalytic small subunits (SSs) activates catalytic activity. The significance for the Cys-12 residue for other plant enzymes has not been investigated. In this study, we replaced the Cys-12 residues with Ala to remove the S-S bond in both *Arabidopsis* and potato AGPase SSs. These $SS_{C12A}$s were co-expressed with the corresponding wild type large subunits (LSs) and the resulting enzymes purified and studied in detail. The elimination of the S-S bond, by either site directed mutagenesis or treatment with a reducing agent, led to an increased affinity for activator and substrate compared to the oxidized wild type enzymes. Increased thermal instability and lower secondary structure was observed for $SS_{C12A}$ containing potato and *Arabidopsis* enzymes. Our results indicated that the S-S bond in *Arabidopsis* is also critical for controlling enzyme's affinity to activator and substrate as well as the enzyme stability at high temperatures.

**Introduction**

The higher plant ADP-glucose pyrophosphorylase (EC 2.7.7.27; AGPase) is an allosterically regulated enzyme that catalyzes the first committed step of starch biosynthesis (Ballicora et al., 2004). In most instances, 3-phosphoglycerate (3-PGA) and inorganic phosphate (Pi) are the main regulators of the higher plant AGPase; however, some seed AGPases, such as those found in wheat and barley endosperm, are not regulated by 3-PGA and Pi (Duffus et al., 1992 and Kleczkowski et al., 1993). Results from several studies provide ample support that the allosteric regulation of AGPase plays a key role in controlling starch biosynthesis (Preiss et al., 1989 and Ballicora et al., 2003). Thus, various attempts have been made to modify the enzyme's allosteric regulatory properties in order to enhance starch production and crop yield (Tjaden et al., 1998, Loef et al., 1999 and Regiere et al., 2002).

Numerous cellular processes are regulated by redox potential in both animal and plant systems (Bucchanan et al., 2005). In plants, redox regulation was first identified in photosynthetic primary carbon metabolism involving the redox proteins ferredoxin and thioredoxin (Bucchanan et al., 1967). The reduction of disulfide bonds is mediated by thioredoxin which, in turn, is reduced by a ferredoxin-thioredoxin reductase in photosynthetic tissue. In nonphotosynthetic tissues, such as potato tuber and other storage organs, thioredoxin is reduced by ferredoxin-NADP reductase (Bucchanan et al., 2005). Therefore, redox regulation is critical not only in leaf tissues where reducing power is generated by electron transport from photosynthesis, but also in storage organs where NADPH is generated from oxidative pentose phosphate pathway.

In addition to allosteric regulation by intermediates of the carbon assimilatory pathway, the plant AGPases are regulated by redox potential (Geigenberger et al., 2005). Redox regulation of plant AGPases have been studied at the biochemical and physiological levels (Fu et al., 1998, Ballicora et al., 2000, Tiessen et al., 2002, Hendriks et al., 2003 and Geigenberger et al., 2005). The recombinant AGPase was activated by reducing agent dithiothreitol (DTT) (Fu et al., 1998). This result implied the existence of a disulfide bond or participation of reducing molecules for enzyme regulation. A conserved N-terminal Cys residue (Cys-12) was identified, which forms an intermolecular disulfide bond (S-S bond) between the potato tuber AGPase SSs (Fu et al., 1998). The reduction of the Cys-12 disulfide bond activated the catalytic activity of AGPase, while oxidation inactivated the enzyme. It has been reported that plant AGPases from pea leaf, potato tuber, and *Arabidopsis* leaf control the starch biosynthesis through redox regulation in response to light and sugars (Hendriks et al., 2003, Kolbe et al., 2005 and Lunn et al., 2006); however, direct evidence for the involvement of Cys-12 residue in these enzymes has not been demonstrated *in planta*.

In order to investigate the role of redox regulation of AGPase, the Cys-12 residue was replaced by Ala (C12A) in both potato tuber and *Arabidopsis* AGPase SS. The $SS_{C12A}$ mutants were co-expressed with AGPase LSs and enzymes purified for biochemical and kinetic analysis. Our results also indicate that the disulfide bond is critical for enzyme stability and fine tune down-regulating enzyme activity by decreasing the apparent affinity of activator and substrate in both potato and *Arabidopsis* enzymes.

**Materials and Methods**

**Materials**

α-D-[$^{14}$C]-glucose -1-phosphate was purchased from Moravek Biochemicals, Inc. *Pfu* Turbo DNA polymerase was purchased from Stratagene. All other chemicals were purchased from Sigma-Aldrich Chemical Co. at the highest commercial grade.

**Site directed mutagenesis**

Cys-12 residue of the *Arabidopsis* AGPase SS cDNA cloned in pHI84 was replaced with Ala by site-directed mutagenesis (QuikChange Mutagensis Kit, Stratagene) to yield the small subunit SS$_{C12A}$. The primers used for site-directed mutagenesis were AraAlaF 5' tct caa aac tct caa act gct ctc gat cct gat gct agc 3', and AraAlaR 5' gct agc atc agg atc gag agc agt ttg aga gtt ttg aga 3'. A similar mutation was inserted in the potato tuber AGPase SS cloned in pSH404 using primers PotAlaF 5' tcg cag aat tca cag aca gct cta gac cca gat gct agc 3', and PotAlaR 5' gct agc atc tgg gtc tag agc tgt ctg tga att ctg cga 3'. PCR was performed using 16 cycles of the following program: 95 °C for 30 sec, 56 °C for 1 min, and 68 °C for 14 min. After digestion with *Dpn* I for 1 hour at 37 °C, the amplified DNA was transformed into competent cells of *Escherichia coli (E. coli)* XL-10-Gold $^{TM}$ (Stratagene).

**Expression and purification of selected AGPases**

Various combinations of LS and SS cDNAs (Figure 1) were co-transformed into *E. coli* EA345 which lacks endogenous AGPase activity. Cells were induced by the addition of 0.1mM IPTG when growth reached OD$_{600}$ of 1.2 to 1.4 at 25 °C and grown for another 24 h. Cells were harvested by centrifugation and the resulting cell pellet resuspended in lysis buffer (0.1M HEPES pH 8.0 and 10% glycerol) containing 200 µg/ml of lysozyme and 1 mM PMSF. The resuspended cells were incubated on ice for 30

min and then were disrupted by sonication.  Cell debris was removed by centrifugation at 12,000g for 10 min, the supernatant collected and then the enzyme subjected to two different purification protocols.  All purification steps were performed at 4 °C.

**Method A**: The clarified cell extracts were loaded onto a DEAE-Sepharose Fast Flow (Amersham) (25 mL of bed volume) which was pre-equilibrated with buffer A (25 mM HEPES pH 8.0 and 5 % glycerol).  The column was washed with buffer A until $OD_{280}$ of the solution fell close to zero.  Proteins were eluted with a linear gradient containing 0 to 0.5 M NaCl in buffer A.  Fractions containing enzyme activity were pooled and then directly loaded onto an immobilized metal affinity column (IMAC) (TALON$^{TM}$ Superflow resin, Clontech) pre-equilibrated with IMAC binding buffer (buffer A and 0.3 M NaCl).  After washing with IMAC wash buffer (IMAC binding buffer and 5 mM imidazole pH 7.5), the proteins were eluted with 0.15 M imidazole pH 7.5 in IMAC binding buffer.  Each fraction was analyzed by SDS-PAGE using a 12 % polyacrylamide gel.  Fractions containing AGPase were collected and precipitated with 67 % ammonium sulfate and left on ice for 3 h.  Protein was collected by centrifugation at 20,000g for 10 min at 4 °C, and resuspended in 2 mL of buffer A, followed by centrifugation at 12,000g for 10 min at 4 °C to remove denatured proteins.  The supernatant was collected and slowly added to 30 mL of buffer A to reduce the salt concentration of the sample.  The enzyme solution was then loaded onto a POROS 20HQ column (1 mL of bed volume) and washed with buffer A followed by 0.15 M NaCl,  The enzyme was eluted with 0.3 M NaCl in buffer A, pooled and concentrated by ultrafiltration (cut-off MW 10 kDa; Milipore) whereupon it was aliquoted and stored at -80 °C until analysis.  The purity of the enzyme was assessed by SDS-PAGE.

**Method B**: AGPase was purified as described in method A except that IMAC purification step was not employed.

**Kinetic study**

ADP-glucose synthesis reaction was performed at 37 °C in 0.1 mL of 100 mM HEPES-NaOH, pH 7.0, 7 mM $MgCl_2$, 3 mM DTT, 5 mM 3-PGA, 0.4 mg/mL bovine serum albumin (BSA), 0.15 U inorganic pyrophosphatase (Sigma), 1.5 mM ATP, 1.5 mM Glc-1-P, and [$^{14}$C] Glc-1-P (1000-1200 dpm/nmol) as described in Hwang et al.(2004).

Kinetic parameters were obtained by fitting the experimental data, which were plotted as velocity versus concentration of substrates or effectors, using the software GOSA (BIO-LOG, B.P. 27201, 31672 LABEGE Cedex, and France). The apparent $A_{0.5}$, and $S_{0.5}$ for 3-PGA and substrates, respectively, correspond to the concentration of these molecules required for the enzyme activity to attain 50% of maximum activity. The $I_{0.5}$ is the amount of Pi required to inhibit the enzyme activity by 50%. One unit (U) of enzyme activity is defined as the amount of enzyme that produces 1 μmol of ADP-glucose in 1 min.

**Heat treatment**

Each enzyme (50 μL per enzyme) in buffer A was incubated at various temperatures (37 , 42, 48, and 60 °C ) for 5 min and then immediately placed on ice for 1 min followed by centrifugation at 12,000g for 10 min at 4 °C. Heat treatment was done under oxidized condition. The supernatants (10 μL per each reaction) were used for enzyme assay in the ADP-glucose synthesis direction under reduced condition.

**Circular dichroism (CD) spectroscopy**

The near homogeneously purified enzymes were selected for CD scanning analysis. The enzyme storage buffer A was replaced by 50 mM ammonium bicarbonate (pH 7.5) as described in Hwang et al., (2007). Wild type and variant AGPases at 1.1 μM were used for CD analysis (AVIV stopped flow CD spectrometer, model 202SF; Protein Solution, Inc.). CD scanning was performed from 300 nm to 200 nm for 3 sec at each wavelength at 25°C and 60°C.

## Results

### Generation and purification of AGPases containing SS$_{C12A}$ and hybrid AGPases

In order to study the significance of the inter-SS disulfide bond in AGPase regulation and stability, the Cys-12 residues of the potato tuber and *Arabidopsis* AGPase SS were replaced by Ala and the resulting SS$_{C12A}$s were co-expressed with the corresponding LSs and then subjected to enzyme purification.

Previously, the Romeo group (Crevillen et al., 2003) reported that the *Arabidopsis* SS was poorly expressed in bacterial cells (*E. coli* AC70-504R), and thus required substantial amount of cells (from 10 L of culture) for enzyme purification and characterization (Crevillen et al., 2003). In this study, we used a new bacterial host cell (*E. coli* EA345) which has a number of advantages over the AC70 strain routinely used in the heterologous AGPase expression (Hwang et al., 2007). Similar to the potato tuber AGPase, the wild type *Arabidopsis* AGPase was purified to near homogeneity using purification method A (results not shown). However, both the potato tuber and *Arabidopsis* SS$_{C12A}$ containing enzymes (PotSS$_{C12A}$/PotLS$_{WT}$ and AraSS$_{C12A}$/AraLS$_{WT}$) did not bind to the IMAC column. The C12A mutation likely resulted in a structural

change altering the accessibility of the His-tagged N-termini of the LSs. Therefore, these $SS_{C12A}$ containing enzymes were only partially purified using method B which eliminated the IMAC purification step (Figure 1). In addition to these enzymes, the potato tuber SS and *Arabidopsis* leaf LS hybrid enzymes ($PotSS_{WT}/AraLS_{WT}$ and $PotSS_{C12A}/AraLS_{WT}$) were also generated for this study as yeast two hybrid analysis showed that *Arabidopsis* LS is a better binding partner for the potato tuber SS than the *Arabidopsis* SS (Kavakli et al., 2002).

The redox state of the purified enzymes was assessed by PAGE. The wild type SS was detected as a dimer by the immuno-blot analysis when performed in the nonreducing SDS-PAGE because of the intermolecular disulfide bond between the SSs (Ballicora et al., 2000). However, the $SS_{C12A}$ mutants were detected as monomers by the immuno-blot analysis (Result not shown).

**Kinetic properties of AGPases containing $SS_{C12A}$ and hybrid AGPases**

The allosteric regulatory properties of the six enzymes were studied using the ADP-glucose synthesis assay. The addition of 3 mM DTT to each enzyme reaction mixture before enzyme assay was considered as the reduced condition, while the absence of DTT in the reaction mixture was regarded as the oxidized condition. The reduced and oxidized forms of the enzymes were detected by immuno-blot analysis using an antibody raised against potato tuber SS (Figure 2).

The apparent $A_{0.5}$ for 3-PGA activation of selected AGPases is shown in Table 1. In each case, the reduced wild type enzymes had lower $A_{0.5}$ values than the oxidized enzyme forms. By contrast, there was no significant difference of $A_{0.5}$ values between

reduced and oxidized forms of $SS_{C12A}$ containing enzymes which mimic the reduced enzyme. $I_{0.5}$ values were also consistent with the apparent affinity of activator. The reduced forms of wild type enzymes were more resistant to the inhibitor Pi (up to 2.3 fold) than oxidized form of enzymes except $SS_{C12A}$ containing enzymes were highly resistant to Pi inhibition in the presence of 2 mM 3-PGA in both reduced and oxidized conditions compared to the wild type enzyme forms (Table 1). The potato and *Arabidopsis* hybrid enzymes showed higher sensitivity to the activator 3-PGA and increased resistance to the inhibitor Pi than wild type enzymes under both reduced and oxidized conditions (Table 1).

The extent of 3-PGA activation or fold-activation for each enzyme is shown in Table 2. The fold-activation was calculated from $V/V_0$ where the V values were measured in the ADP-glucose synthesis direction in the presence of 1 mM 3-PGA while the $V_0$ values were obtained in the absence of 3-PGA. Both potato and *Arabidopsis* wild type enzymes were activated to a greater extent (2.4- to 2.6-fold) by 3-PGA in the reduced condition than in the oxidized condition. The extent of activation by 3-PGA for the $SS_{C12A}$ containing potato and *Arabidopsis* enzymes, however, showed less differences in 3-PGA fold activation depending on the redox state. The reduced form of the $AraSS_{C12A}/AraLS_{WT}$ enzyme showed less fold-activation (0.7-fold) by 3-PGA than the oxidized form while the reduced form of the corresponding potato enzyme, $PotSS_{C12A}/PotLS_{WT}$, was slightly more activated by 3-PGA (1.2-fold) than the oxidized form. Interestingly, the wild type hybrid $PotSS_{WT}/AraLS_{WT}$ enzyme showed a somewhat larger fold-activation for 3-PGA in the oxidized state than in the reduced state (1.3 fold)

while the C12A containing hybrid enzyme, PotSS$_{C12A}$/AraLS$_{WT,}$ showed a definite larger fold-activation for the reduced enzyme than oxidized form (1.8 fold).

The apparent substrate affinity constants for all six enzymes were also studied under oxidized and reduced conditions. In the reduced state, the apparent affinity for ATP of the wild type *Arabidopsis* and hybrid enzymes was higher (up to 2.7-fold) than under the oxidized condition, whereas the C12A versions of these enzymes showed no significant differences. The potato wild type enzyme showed only a marginal increase (up to 1.3 fold) in affinity for ATP in the reduced state than in the oxidized state. The apparent affinity for ATP for the SS$_{C12A}$ containing potato and *Arabidopsis* enzymes were similar under both conditions (less than 1.3 fold). Interestingly, the apparent affinity for glucose-1-phosphate was not significantly affected by the redox state of these enzymes. $V_{max}$ and $K_{cat}$ values for these enzymes were also similar under both oxidized and reduced conditions (less than 1.5 fold) (Table 3).

**Heat stability of wild type and SS$_{C12A}$ containing enzymes**

In order to investigate the significance of the S-S bond on enzyme stability, the catalytic activity of the enzymes was measured after pretreatment at various temperatures. Figure 3 shows that SS$_{C12A}$ containing enzymes were more susceptible to elevated temperatures than wild type enzymes. This result indicates that the S-S bond of both potato tuber and *Arabidopsis* AGPase SSs is also critical for maximum heat stability.

To investigate the physical state of the enzyme at a high temperature, circular dichroism (CD) analysis was performed at 25 °C and 60 °C with potato tuber wild type enzyme (PotSS$_{WT}$/PotLS$_{WT}$), and potato and *Arabidopsis* hybrid enzymes

(PotSS$_{WT}$/AraLS$_{WT}$, and PotSS$_{C12A}$/AraLS$_{WT}$).   The other SS$_{C12A}$ containing enzymes were not included in the CD analysis because they were not pure.  The CD analysis showed that at higher temperature, AGPases had less secondary structure and that the loss of the small subunit disulfide bond increases this reduction in secondary structure at elevated temperatures (Figure 4).  These kinetic and biochemical studies indicate that the S-S bond is critical for maintaining secondary structure of AGPase to help the enzyme to keep correctly folded structures at a higher temperature.


**Discussion**

**The disulfide bond affects the apparent affinity for effectors**

Purified recombinant *Arabidopsis* AGPase has never been examined for redox regulation at the biochemical level.  Here, we generated SS$_{C12A}$ mutants lacking a disulfide bond in both potato tuber and *Arabidopsis* leaf AGPases, thereby mimicking reduced AGPases.  Potato and *Arabidopsis* hybrid enzymes were also studied because of the poor expression of *Arabidopsis* SS and stronger interaction between *Arabidopsis* LS and potato tuber SS than *Arabidopsis* LS and SS (Kavakli et al., 2002).

The potato tuber and *Arabidopsis* wild type enzymes have different allosteric properties depending on the redox states.  Both the *Arabidopsis* and potato tuber wild type enzymes had higher affinity for the activator 3-PGA in the reduced condition than in the oxidized state.   SS$_{C12A}$ containing enzymes (PotSS$_{C12A}$/PotLS$_{WT}$ and AraSS$_{C12A}$/AraLS$_{WT}$) showed no differences in the apparent affinity for 3-PGA under reducing and oxidizing conditions with A$_{0.5}$ values nearly identical to those obtained for the wild type enzymes under reducing conditions.  Hence, this specific Cys-12 disulfide

bond is directly responsible for the lower affinity of the enzymes toward the activator 3-PGA. Both wild type enzymes were more resistant to inhibitor Pi under reduced condition than oxidized state. By contrast, the $SS_{C12A}$ containing enzymes showed increased resistance to Pi inhibition irrespective of the redox condition. This result indicates that Cys at position 12 is essential for normal regulatory response to the inhibitor Pi. Interestingly, removal of the first 12 amino acids including the redox-sensitive Cys from the SS N-terminus renders the enzyme with up-regulatory properties, i.e. increased sensitivity to the activator 3-PGA and increased resistance to Pi inhibition (Laughlin et al., 1998). The presence of the N-terminal peptide is essential for normal allosteric regulatory response by these AGPases.

The reduced condition enables AGPase to bind ATP more efficiently than under the oxidized condition. The $S_{0.5}$ values for glucose-1-phosphate (Glc-1-P), however, were only slightly altered in the reduced and oxidized states (less than 1.3 fold). This relatively minor change in the apparent affinity for substrates (for both ATP and Glc-1-P) has been reported for the potato tuber wild type AGPase under both oxidized and reduced conditions (Ballicora et al., 2000).

Our results showed $SS_{C12A}$ containing enzymes have the same catalytic properties as the reduced enzymes. There is no significant difference in $V_{max}$ and $K_{cat}$ values for these enzymes (less than 1.5 fold). The reduction of AGPase affects the affinity of effectors rather than enzyme catalysis.

In this study, we showed that the potato tuber SS and *Arabidopsis* LS hybrid enzymes ($PotSS_{WT}/AraLS_{WT}$ and $PotSS_{C12A}/AraLS_{WT}$) were highly sensitive to the activator 3-PGA irrespective of their reduction and oxidation states. Likewise, Ventriglia

et al (2007) have recently reported that the potato tuber and *Arabidopsis* hybrid enzymes have higher sensitivity to the activator 3-PGA than each corresponding wild type enzymes in the reduced state (Ventriglia et al, 2007). Ventriglia et al (2007) reported that reduced form of wild type potato and *Arabidopsis* enzymes were activated by 3-PGA up to 30 and 80 fold respectively, and PotSS$_{WT}$/AraLS$_{WT}$ enzyme was activated by 3-PGA about 4.4 fold in the reduced condition. In our results, the potato tuber SS and *Arabidopsis* LS hybrid enzymes were also less activated by 3-PGA than wild type enzymes (up to 6 fold) at the presence of 1 mM 3-PGA in the reduced conditions (Table 2).

Both potato and *Arabidopsis* SS$_{C12A}$ containing enzymes were less activated by 3-PGA than wild type enzymes in the reduced condition while the activation fold by 3-PGA in the oxidized form of enzymes was seen for mild differences between wild type and SS$_{C12A}$ containing enzymes. This result indicated that the disulfide bond is important for enzyme regulation by 3-PGA through modulating 3-PGA mediated enzyme activation. The oxidized form of SS$_{C12A}$ containing hybrid enzyme (PotSS$_{C12A}$/AraLS$_{WT}$) was less activated by 3-PGA than PotSS$_{WT}$/AraLS$_{WT}$ about 4 fold comparing with reduced form of PotSS$_{C12A}$/AraLS$_{WT}$ enzyme which showed 1.9 fold less 3-PGA activation fold than PotSS$_{WT}$/AraLS$_{WT}$ enzyme (Table 2). The different 3-PGA activation pattern in hybrid enzymes comparing to the wild type enzymes suggests that enzyme structure or subunit interaction may be changed by distinct subunit types from different plant species. Therefore hybrid enzymes have a greater effect on the affinity of effector rather than the enzyme activation by effector.

**The disulfide bond is required for maintaining ordered structure**

Most of the AGPases characterized thus far, are stable at higher temperatures (up to 60 °C) except for several monocot endosperm AGPases (Ballicora et al., 1999). The AGPase from photosynthetic bacteria requires Pi for enzyme's thermal stability at 60 °C, but both potato tuber and *Arabidopsis* AGPase do not need Pi for enzyme stability at high temperatures (Ballicora et al., 1999). The reduced potato tuber AGPase has been characterized in terms of heat stability (Ballicora et al., 1999). The SS with truncated N-terminus and $SS_{C12A}$ mutant enzymes completely lost their enzyme activity at 60 °C while wild type enzyme had about 50% remaining enzyme activity (Ballicora et al., 1999). This result indicated that the reduction of disulfide bond between Cys-12 residues of potato tuber SSs is responsible for the loss of enzyme activity, and the disulfide bond is important for heat stability in the potato tuber AGPase. Additional evidence has been reported from the study of maize endosperm AGPase (Linebarger et al., 2005). The QTCL motif containing Cys-12 residue from potato tuber AGPase SS was inserted into the N-terminal residues of heat labile maize endosperm AGPase SS. The heat stability was dramatically enhanced in the maize endosperm AGPase containing the QTCL motif (Linebarger et al., 2005). These results supported that the S-S bond is responsible for enhancing enzyme stability at high temperatures. Other plant AGPases has not been studied regarding redox regulation or enzyme stability using purified enzymes except for the potato tuber AGPase.

The biochemical nature of $SS_{C12A}$ containing enzyme was studied at a higher temperature using circular dichroism (CD) spectroscopy and kinetic analysis. The heat stability of *Arabidopsis* AGPase, and potato-*Arabidopsis* hybrid AGPases was

investigated in this study.  As expected, compared to the oxidized wild type enzymes containing a disulfide bond, the $SS_{C12A}$ containing enzymes were less stable at high temperatures and lost enzyme activity more readily.  This increased sensitivity to heat was also reflected in the structural state of these enzymes.  The $SS_{C12A}$ containing enzyme had less secondary structure than wild type enzyme when temperature was increased from 25 °C to 60 °C.  These results indicate that the S-S bond is responsible for enzyme stability by maintaining the enzyme structure.  Detailed structural analysis of these enzymes at different pH and in the presence of substrates is required in order to understand structural changes at a higher temperature.

In order to investigate the role of redox regulation of *Arabidopsis* AGPase in controlling starch synthesis *in planta*, we have constructed a plasmid that containing the *Arabidopsis* genomic $SS_{C12A}$ gene and transformed it into *Arabidopsis* TL25 which contains an inactivated AGPase SS gene.  The study of these transgenic plants, which are currently being selected, will provide direct evidence on the role of redox regulation of this enzyme in carbon partitioning and controlling of starch synthesis *in planta.*

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**Figure legends**

**Figure 1. Purification scheme of various AGPases.**

The six enzymes were purified by two different purification schemes, A and B. Pot: Potato, Ara: *Arabidopsis*, WT: wild type, and C12A: Cys 12 residue was replaced by Ala.

**Figure 2. Immunoblot analysis of AGPases.**

Purified potato tuber wild type AGPases were analyzed by SDS-PAGE using 12% polyacrylamide gels under non-reducing conditions. Samples of lanes 1 and 2 were pretreated in buffer A with 3 mM DTT for 30 min on ice while those in lanes 3 and 4 were placed on ice in the absence of DTT. Lanes 1 and 3 contain 200 ng of protein while lanes 2 and 4 contain 100 ng.

**Figure 3. Heat stability of wild type and disulfide bond mutant enzymes.**

The purified enzymes were pre-incubated at different temperatures as described in method section, and then their enzyme activity measured using the ADP-glucose synthesis assay. The average values of two independent experiments are shown (less than 15% of standard error in each case).
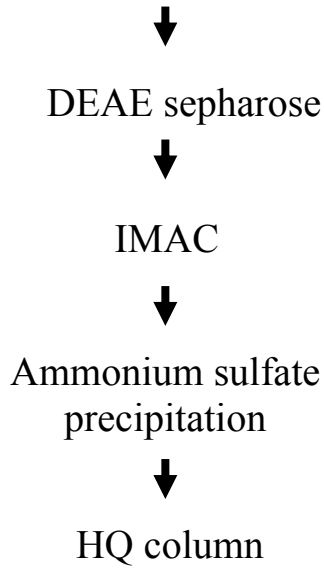
**Figure 4. Circular dichroism (CD) spectra of wild type potato (a), wild type hybrid (b) and C12A containing hybrid enzymes (c) at 25 °C and 60 °C.**

**Figure 1.**

| LS | SS | Purification method |
|---|---|---|
| PotWT | PotWT | A |
| PotWT | PotC12A | B |
| AraWT | AraWT | A |
| AraWT | AraC12A | B |
| AraWT | PotWT | A |
| AraWT | PotC12A | A |

**Method A**
Crude extract (1L expression)

↓

DEAE sepharose

↓

IMAC

↓

Ammonium sulfate
precipitation

↓

HQ column

**Method B**
Crude extract (1L expression)

↓

DEAE sepharose

↓

Ammonium sulfate
precipitation

↓

HQ column
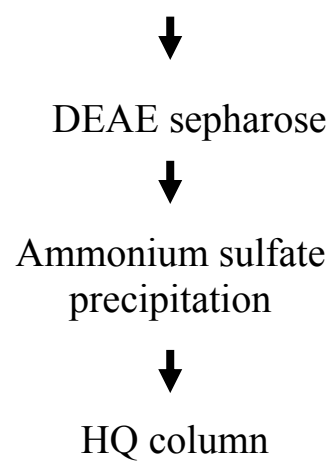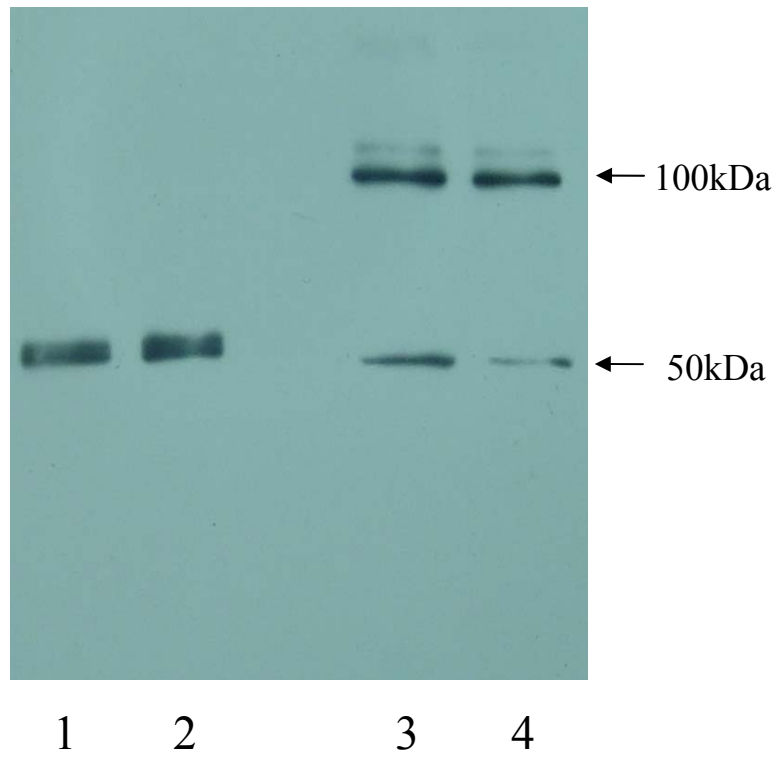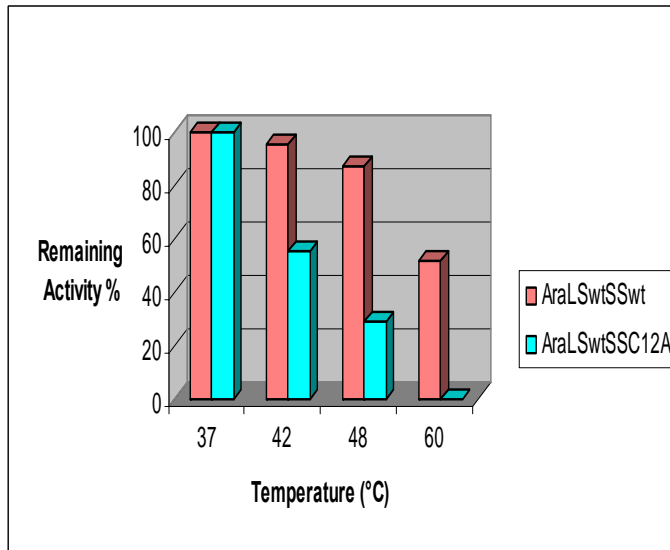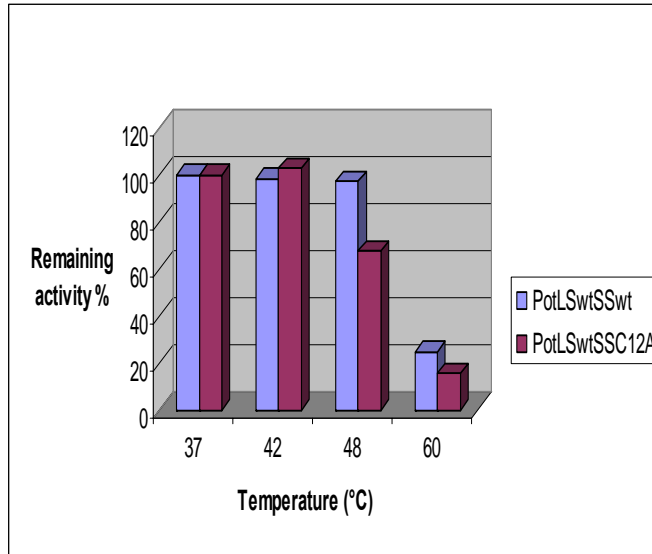
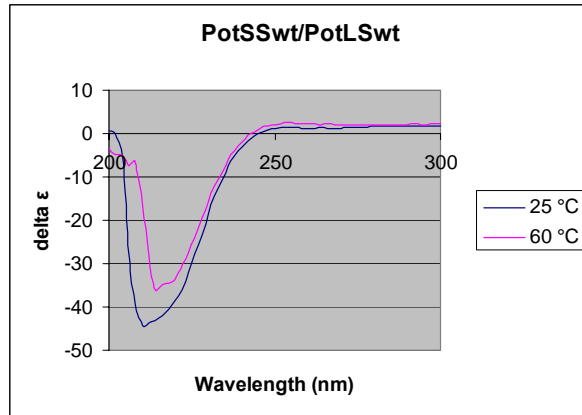**Figure 2.**
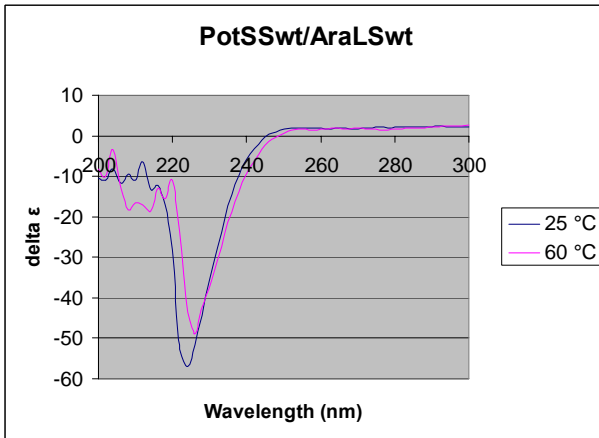
**Figure 3.**

**Figure 4-a)**
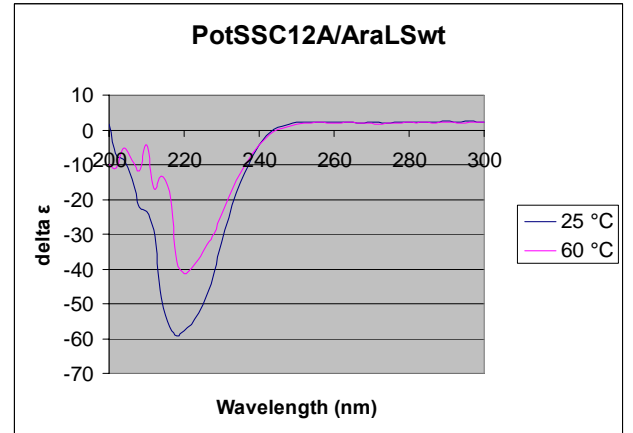


**Figure 4-b)**

**Figure 4-c)**

**Table 1. Allosteric regulatory parameters of selected AGPases.**

Kinetic values were obtained by assaying the various enzymes using the ADP-glucose synthesis direction. The mean values (in mM) have a standard error of less than 15% in all cases. WT denotes wild type.

| LS | PotWT | PotWT | AraWT | AraWT | AraWT | AraWT |
|---|---|---|---|---|---|---|
| SS | PotWT | PotC12A | AraWT | AraC12A | PotWT | PotC12A |
| $A_{0.5}$ (mM) for 3-PGA | | | | | | |
| Oxidized | 0.33 | 0.11 | 0.27 | 0.04 | 0.04 | 0.01 |
| Reduced | 0.11 | 0.07 | 0.07 | 0.05 | 0.02 | 0.01 |
| $I_{0.5}$ (mM) @ 2 mM 3-PGA | | | | | | |
| Oxidized | 2.2 | > 10 | 2.8 | 7.5 | 5.2 | > 10 |
| Reduced | 5 | > 10 | 3.5 | 8.2 | 9.5 | > 10 |

**Table 2. The fold activation by 3-PGA.**

The fold activation was calculated from $V/V_0$. The V values were obtained in the ADP glucose synthesis reaction with the presence of 1 mM 3-PGA, and the $V_0$ values were obtained in the absence of 3-PGA.

| LS | PotWT | PotWT | AraWT | AraWT | AraWT | AraWT |
|---|---|---|---|---|---|---|
| SS | PotWT | PotC12A | AraWT | AraC12A | PotWT | PotC12A |
| Oxidized | | | | | | |
| $V_0$ (U/mg) | 2.0 | 0.008 | 1.30 | 0.005 | 0.09 | 0.28 |
| V (U/mg) | 22.3 | 0.120 | 21.4 | 0.090 | 1.30 | 0.90 |
| $V/V_0$ (Fold) | 11.1 | 15 | 16.5 | 18 | 14.4 | 3.2 |
| Reduced | | | | | | |
| $V_0$ (U/mg) | 0.8 | 0.01 | 0.5 | 0.011 | 0.12 | 0.12 |
| V (U/mg) | 22.7 | 0.18 | 20.2 | 0.13 | 1.35 | 0.69 |
| $V/V_0$ (Fold) | 28.4 | 18 | 40.4 | 12 | 11.3 | 5.8 |

**Table 3. Kinetic parameters of selected AGPases.**

Kinetic values were obtained by assaying the various enzymes using the ADP-glucose synthesis direction. The mean values (in mM) have a standard error of less than 15% in all cases. WT denotes wild type.

| LS | PotWT | PotWT | AraWT | AraWT | AraWT | AraWT |
|---|---|---|---|---|---|---|
| SS | PotWT | PotC12A | AraWT | AraC12A | PotWT | PotC12A |
| $S_{0.5}$ (mM) for ATP | | | | | | |
| Oxidized | 0.16 | 0.10 | 0.24 | 0.09 | 0.40 | 0.19 |
| Reduced | 0.12 | 0.08 | 0.09 | 0.08 | 0.22 | 0.20 |
| $S_{0.5}$ (mM) for Glc-1-P | | | | | | |
| Oxidized | 0.13 | 0.20 | 0.13 | 0.07 | 0.09 | 0.07 |
| Reduced | 0.11 | 0.18 | 0.10 | 0.08 | 0.08 | 0.08 |
| Vmax (U/mg) | | | | | | |
| Oxidized | 35.9 | 0.12 | 21.4 | 0.09 | 1.30 | 0.90 |
| Reduced | 34.7 | 0.18 | 20.2 | 0.13 | 1.35 | 0.69 |
| Kcat (1/s) | | | | | | |
| Oxidized | 120 | 0.40 | 71.5 | 0.30 | 4.3 | 3.0 |
| Reduced | 116 | 0.60 | 67.5 | 0.43 | 4.5 | 2.3 |