Biochem. J. (1992) 287, 821–825 (Printed in Great Britain)

Purification and properties of a phosphatase in French bean (*Phaseolus vulgaris* L.) leaves that hydrolyses 2′-carboxy-D-arabinitol 1-phosphate

Alison H. KINGSTON-SMITH, Iain MAJOR, Martin A. J. PARRY and Alfred J. KEYS*
Agricultural and Food Research Council, Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, U.K.

An enzyme that releases P_i from 2-carboxy-D-arabinitol 1-phosphate, a naturally occurring tightly binding inhibitor of ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), was purified from leaves of French bean seedlings. It was a monomeric protein of M_r about 56000. Catalytic activity was stimulated by increased concentrations of inorganic salts to a maximum at an ionic strength above 0.2. NADPH and D-fructose 1,6-bisphosphate increased the activity of the enzyme in both the presence and absence of 0.2 M-KCl. The pure enzyme did not require dithiothreitol for activity. The pH optimum was 7, the K_m for 2-carboxy-D-arabinitol 1-phosphate was 0.43 mM and the specific activity 6.8 μmol/min per mg of protein. The enzyme had little or no activity against phosphate ester intermediates of photosynthetic metabolism and glycolysis but hydrolysed the 1,5-bisphosphates of 2′-carboxy-D-ribitol and 2′-carboxy-D-arabinitol more rapidly than 2′-carboxy-D-arabinitol 1-phosphate.

INTRODUCTION

In many plant species (Seemann *et al.*, 1990), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is inhibited in the leaves at night by a tightly binding inhibitor identified as 2′-carboxy-D-arabinitol 1-phosphate (CA1P) (Gutteridge *et al.*, 1986; Berry *et al.*, 1987). The inhibition disappears when the leaves are illuminated (Vu *et al.*, 1983) and hence a diurnal variation in Rubisco activity in extracts is observed. An alternative name for CA1P is 2′-phospho-D-hamamelonic acid (Beck *et al.*, 1989) and the synthesis of D-hamamelose 2′,5-bisphosphate in spinach chloroplasts from CO_2 (Beck *et al.*, 1971) suggests a potential synthetic pathway. However, more progress has been made in the search for enzymes involved in the breakdown of CA1P than in its synthesis.

An enzyme present in the leaves of tobacco was initially reported to destroy CA1P by a reaction dependent on NADPH (Salvucci *et al.*, 1988). Later this enzyme was identified as a phosphatase that hydrolysed CA1P and which was activated by NADPH (Holbrook *et al.*, 1989; Gutteridge & Julien, 1989). A protein fraction from leaves of French beans (*Phaseolus vulgaris*) destroyed the inhibitory activity of CA1P towards Rubisco and caused a release of P_i (Keys *et al.*, 1989). We describe a simple purification of the enzyme responsible and some of its properties. Similarities to the CA1P phosphatase in tobacco leaves are discussed together with properties that need to be taken into account in further studies.

EXPERIMENTAL

Materials

French-bean (cv. Tendergreen) seed was sown in compost in trays (35 cm × 21 cm × 7.5 cm each with 40 seeds) and the plants were grown for 2–3 weeks in a glasshouse with 20 °C day, 16 °C night temperatures and with the daylength extended as necessary to 16 h with artificial lighting. Leaves were cut from the plants in the morning after at least 2 h of light and immersed in cold (< 5 °C) distilled water. The accumulated leaves were taken to a room at 0–5 °C for immediate use.

Ribulose 1,5-bisphosphate (RuBP) was prepared from AMP (Wong *et al.*, 1980) and purified by anion-exchange chromatography using a LiCl gradient in 3 mM-HCl (Pierce *et al.*, 1980). 2′-Carboxy-D-arabinitol 1,5-bisphosphate (CABP) and 2′-carboxy-D-ribitol 1,5-bisphosphate (CRBP) were made from RuBP by reaction with cyanide under weakly alkaline conditions and separated using the methods described by Pierce *et al.* (1980). CA1P was made from CABP by treatment with potato acid phosphatase until 50% of the organic phosphorus had been released as P_i (Gutteridge *et al.*, 1989). The CA1P was separated from the P_i and purified by chromatography on DEAE-Sephacel using a linear gradient in NH_4HCO_3. Phosphate esters were stored at −25 °C as frozen aqueous solutions of the sodium salts dispensed into 1.5 ml microfuge tubes; the sodium contents were such that, before freezing, the solutions of RuBP were pH 6.5 and the solutions of carboxypentitol phosphates were between pH 7 and 8 to avoid excessive lactone formation. The final concentrations of phosphate esters were determined from the total phosphorus content. For this, samples of each stock solution were heated with $MgNO_3$, and the P_i released from the residue by heating in dilute acid (Ames, 1966) was measured (van Veldhoven & Mannaerts, 1987). For RuBP, the concentration was confirmed by measuring the amount of $^{14}CO_2$ fixed when a sample of the stock solution was incubated with pure activated Rubisco in the presence of 10 mM-$NaH^{14}CO_3$. Inhibition of Rubisco activity was used to confirm concentrations of the 2′-carboxy-D-arabinitol phosphates.

Assay for phosphatase activity during purification

Samples containing protein were incubated at 25 °C in 50 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/10 mM-$MgCl_2$/1 mM-$CaCl_2$/

Abbreviations used: CABP, 2-carboxy-D-arabinitol 1,5-bisphosphate; CA1P, 2-carboxy-D-arabinitol 1-phosphate; CRBP, 2-caroboxy-D-ribitol 1,5-bisphosphate; RuBP, D-ribulose 1,5-bisphosphate; Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; FBP, D-fructose 1,6-bisphosphate; DTT, dithiothreitol.

* To whom correspondence should be addressed.

10 mM-dithiothreitol (DTT)/1 % (w/v) BSA/0.5 mM-CA1P in a total volume of 50 μl. Reaction was started by addition of enzyme and stopped after an appropriate time by the addition of 2.5 μl of trifluoroacetic acid. After 10 min, the precipitated protein was removed by centrifugation for 5 min at 10000 *g*. The supernatant was transferred to polystyrene test tubes (1.2 × 7.5 cm) containing 0.95 ml of water. P_i was estimated in the diluted sample by the method of van Veldhoven & Mannaerts (1987).

Purification of the enzyme

Insoluble polyvinylpyrrolidone (8 g) was added to 800 ml of 20 mM-Tris/HCl, pH 8.0, containing 20 mM-$MgCl_2$ and 1 mM-$CaCl_2$. The suspension was cooled to 0–5 °C overnight. After gassing the suspension with N_2 for 5 min, the following were added: 3.2 g of casein, 800 μmol of phenylmethanesulphonyl fluoride in 2 ml of ethanol, 800 μmol of *p*-aminobenzamidine, 80 nmol of pepstatin A and 2.8 ml (40 mmol) of 2-mercaptoethanol. Leaves from eight trays of plants (approx. 400 g fresh weight) were drained and homogenized in the above mixture using a Waring blender for 5 × 15 s with 15 s rests. The homogenate was filtered through four layers of muslin and the filtrate left to stand for 10 min at 0–5 °C. Solid $(NH_4)_2SO_4$ was dissolved in the filtrate to 40 % saturation. The precipitate which formed was removed by centrifugation and discarded. Further solid $(NH_4)_2SO_4$ was added to the supernatant to give 60 % saturation. The precipitate was recovered by centrifugation, drained and resupended in 70 ml of homogenization buffer without polyvinylpyrrolidone and casein. This solution was layered above a stepped sucrose gradient in eight polyallomer tubes (Beckman 2.5 cm × 8.2 cm) each containing 2 ml of 1.2 M, 10 ml of 1 M, 8 ml of 0.6 M, 2.5 ml of 0.4 M and 2.5 ml of 0.3 M-sucrose in 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA, 10 mM-$MgCl_2$, 1 mM-$CaCl_2$, 10 mM-$NaHCO_3$ and 2 mM-DTT (buffer A). The tubes were centrifuged in a type 70 Ti angle rotor using an L8-70 ultracentrifuge (Beckman) at 70000 rev./min (371000 $g_{av.}$) for 1.5 h. The layer above the 0.4 M-sucrose solution was recovered leaving the Rubisco mainly in the 0.6 M-sucrose layer. Salts and substances of low M_r were separated from the proteins by gel filtration using a column (5 cm × 40 cm) of Sephadex G-25 (Pharmacia) previously equilibrated with 5 mM-Bicine/NaOH, pH 8.0, containing 0.1 mM-*p*-aminobenzamidine, 0.1 μM-pepstatin A and 50 mM-2-mercaptoethanol. Fractions containing the protein were combined and the solution was pumped on to a column (2.5 cm × 15 cm) of Dyematrex Green A (Amicon Ltd., Stonehouse, Glos., U.K.) equilibrated with buffer A. Proteins were eluted with a 0–1.5 M-KCl gradient in the same buffer at 40 ml/h in 18.75 h. Fractions (10 ml) containing the phosphatase activity were combined and the solution was diluted with an equal volume of buffer A. This dilution is critical to prevent precipitation of K_2SO_4 with the protein in the next step. Protein was precipitated by the addition of solid $(NH_4)_2SO_4$ to give 80 % saturation and redissolved in a minimum volume of buffer A. The solution was loaded on to a Sephacryl S300 (Pharmacia) gel-filtration column (1.3 cm × 73 cm) operated at 5.2 ml/h using buffer A (a Pharmacia Superose 12 f.p.l.c. colum was used as an alternative for this step). The enzyme behaved like a protein of much lower M_r during the gel-filtration step and this behaviour was exaggerated if too much $(NH_4)_2SO_4$ was carried through from the previous step. We believe the explanation is a hydrophobic interaction with the column matrix dependent on salt concentration and local perturbation of pH. Fractions (2.6 ml) containing the phosphatases activity were combined and this solution was loaded on to an f.p.l.c. Mono Q (Pharmacia) anion-exchange column using a Superloop (Pharmacia). The column was operated at room temperature at 1 ml/min with a gradient of 0–0.6 M-KCl in buffer A developed over 35 min and from 0.6 to 1 M-KCl from 35 to 45 min. Fractions (0.5 ml) containing phosphatase activity were combined and dialysed overnight against 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 2 mM-$CaCl_2$. The dialysed solution could be stored at −25 °C for several months without significant loss of activity.

Other methods

Protein content in solutions was determined by the dye-binding method (Bradford, 1976). SDS/PAGE was based on the system described by Laemmli (1970).

RESULTS

Purification

Fig. 1 shows the polypeptides present at various stages of the purification separated by SDS/PAGE and stained with Coomassie Blue. On the basis of its mobility in SDS/PAGE, the final product is a single peptide of M_r close to 56000. Table 1 shows the progress of purification in terms of activity of the phosphatase. The most effective purification step appears to be the Amicon Green A column which resulted in a 25-fold increase

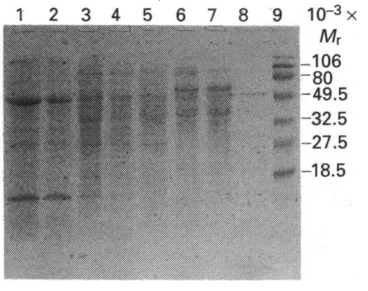

Fig. 1. Polypeptides present at various stages in the purification of CA1P phosphatase from leaves of *Phaseolus vulgaris* L. separated by SDS/PAGE

Lanes 1 and 2, proteins in clarified leaf homogenate (40 μg of total protein in lane 1 and 20 μg in lane 2); lanes 3 and 4, fraction precipitating between 40 and 60 %-satd $(NH_4)_2SO_4$ (40 μg and 20 μg); lane 5, after removal of most of the Rubisco by sucrose-gradient centrifugation (20 μg); lane 6, active fractions from the Dyematrex Green A column (20 μg); lane 7, active fractions from Sephacryl S300 (20 μg); lane 8, protein in the pooled active fractions from the Pharmacia Mono Q f.p.l.c. column (1 μg); lane 9, protein standards, $10^{-3} \times M_r$: 106, 80, 49.5, 32.5, 27.5, 18.5.

Table 1. Recovery of catalytic activity in relation to protein during purification

Activity was determined at pH 7.5 and not under optimal conditions. Where necessary the protein solutions were desalted using Pharmacia G-25 Sephadex.

Purification step	Total protein (mg)	Total activity (nmol/min)	Specific activity (μmol/min per mg of protein)	Purification (fold)
Homogenate	8064	6413	8×10^{-4}	1
$(NH_4)_2SO_4$	1344	3094	2.3×10^{-3}	2.9
Density gradient	545	1285	2.4×10^{-3}	3.0
Amicon Green A	19.6	1161	0.06	75
Superose 12	3.11	1120	0.36	450
Mono Q	0.132	226	1.71	2138

Table 2. Effect of salts and NADPH on the purified enzyme

Additions are shown to a basic reaction mixture containing 50 mM-Tris, pH 7.5, 1 mM-EDTA, 0.5 mM-CA1P and 0.33 μg of protein in a final volume of 50 μl. These are combined results from two separate experiments with different batches of enzyme. Rates were based on time courses.

Addition	Concentration (mM)	Rate of release of P_i (relative)
None	–	100
$CaCl_2$	0.4	67
	0.8	101
	2.0	117
	4.0	132
$MgCl_2$	1.7	94
	3.3	106
	10.0	162
	20.0	213
$(NH_4)_2SO_4$	1.7	110
	3.3	145
	10.0	186
	20.0	240
$NaHCO_3$	10.0	162
KCl	200.0	261
NaCl	200.0	248
NADPH	2.0	252

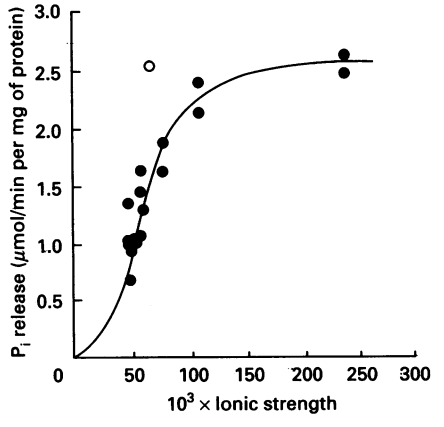

Fig. 2. Dependence of phosphatase activity towards CA1P on the total ionic strength in the reaction mixture

Initial rates of hydrolysis were determined in the presence of 50 mM-Tris/HCl, pH 7.0, with 0.5 mM-CA1P, 10 mM-$MgCl_2$, 2 mM-DTT and 0.33 μg of purified protein. The point marked by an open circle represents activity in the presence of 2 mM-NADPH. Each point represents a rate of hydrolysis in a different ionic environment for which the ionic strength ($0.5\ \Sigma C_i Z_i^2$, where C_i is the molarity of individual ions and Z_i their charge) has been calculated.

Table 3. Effects of DTT, NADPH, $MgCl_2$ and FBP with and without 0.2 M-KCl on CA1P phosphatase activity

All reaction mixtures contained 50 mM-Bistris (methane)/HCl, pH 7, 0.1% BSA, 1 mM-CA1P and 11 μg (experiment A) or 3.3 μg (experiment B) of enzyme protein per ml. The final concentrations of the added components were: 2 mM-DTT, 2 mM-NADPH, 10 mM-$MgCl_2$, 10 mM-FBP and 0.2 M-KCl. Reaction at 25 °C was started by the addition of CA1P. Control reaction mixtures without CA1P or without enzyme produced no P_i except where FBP was present when the enzyme-dependent release was about 1% of that with CA1P. The values are typical of three (experiment A) and five (experiment B) measurements of rate with the same enzyme made at different times. Consistent small trends in values were observed with time of measurement but effects of treatment were the same throughout.

Addition(s)	Release of P_i (μmol/min per mg of protein)
Experiment A	
DTT	0.27
DTT, NADPH	0.75
DTT, KCl	2.43
DTT, NADPH, KCl	3.85
DTT, $MgCl_2$	0.64
DTT, KCl, $MgCl_2$	1.92
DTT, FBP	4.44
DTT, KCl, FBP	6.21
Experiment B	
DTT, KCl	1.76
DTT, NADPH, KCl	3.03
KCl	2.28
NADPH, KCl	2.65

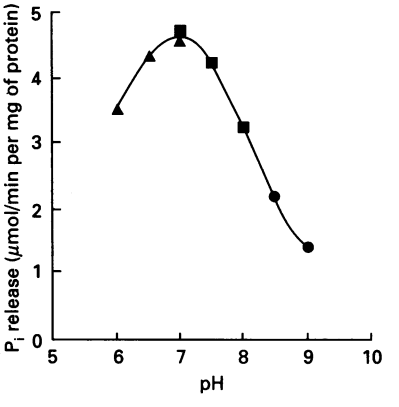

Fig. 3. Dependence on pH of phosphatase activity towards CA1P

Reaction mixtures contained 50 mM-Bistris/HCl (▲), Tris/HCl (■) or Bicine/NaOH (●) at the pH values indicated with 1 mM-EDTA, 10 mM-$MgCl_2$, 10 mM-$(NH_4)_2SO_4$, 20 mM-DTT, 0.1% (w/v) BSA and 0.075 μg of purified phosphatase in a final volume of 50 μl.

in specific activity. Variable losses of activity on the Mono Q column have caused concern but remain largely unexplained.

Effect of salts, DTT, fructose 1,6-bisphosphate (FBP) and NADPH on activity

The effect of the addition of various salts is shown in Table 2. There is a non-specific effect of ions on activity; this is consistent with observations (not included) that we made with impure CA1P phosphatase and crude extracts. In Fig. 2, activity is shown as a function of ionic strength, taking into account buffer ions and added salts. Activity depends on ionic strength with a maximum sensitivity between 0.05 and 0.1 and saturation at about 0.2. NADPH has an activating effect at low ionic strength (Table 2, Fig. 2); it also stimulates activity in the presence of 0.2 M-KCl (Table 3).

Experiments A and B of Table 3 were conducted at pH 7 and with the substrate concentration increased to 1 mM. Under these conditions KCl alone increased activity ninefold and NADPH alone increased activity threefold. When both substances were present the effects were additive. The considerable activation by 10 mM-FBP reported by Holbrook *et al.* (1991) on the CA1P phosphatase from tobacco is found also with the French-bean enzyme. This effect also was additional to the effect of KCl.

Some experiments showed a slight activating effect of DTT in the presence of BSA and these substances were included in

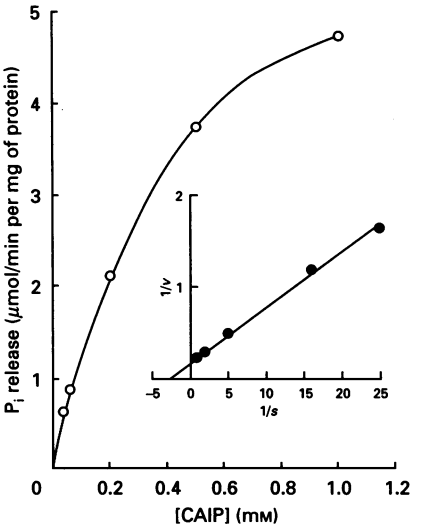

Fig. 4. Dependence of phosphatase activity on the concentration of CA1P

Reaction mixtures contained 50 mM-Tris/HCl, pH 7.0, 0.2 M-KCl, 10 mM-$MgCl_2$, 2 mM-DTT, 0.1% (w/v) BSA and 0.39 μg of purified phosphatase in a volume of 50 μl. The lines were plotted, and the values of K_m = 0.432 mM for CA1P (S.E. 0.026) and $V_{max.}$ = 6.83 μmol/min per mg of protein (S.E. 0.18) were calculated using an IBM compatible computer with ENZFITTER software (Elsevier, Biosoft).

Table 4. Substrate specificity of the phosphatase

Reaction mixtures contained 50 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 10 mM-$NaHCO_3$, 10 mM-$MgCl_2$, 10 mM-$(NH_4)_2SO_4$, 0.1 mg of BSA/ml and 20 mM-DTT with the substrate shown at a concentration of 0.5 mM.

Substrate	Phosphatase activity (relative)
CA1P	100
CABP	432
CRBP	131
RuBP	15
FBP	5
D-3-Phosphoglyceric acid	5
Pyrophosphate	3
Phosphoglycollic acid	1
D-Glucose 1-phosphate	1
D-Glucose 6-phosphate	0
D-Fructose 6-phosphate	0
D-6-Phosphogluconate	0
D-Sedoheptulose 1,7-bisphosphate	0
NADP	0
ATP	0
5′-AMP	0

reaction mixtures for many further experiments together with KCl or another salt to increase the ionic strength. However, Table 3, Experiment B shows that DTT is not required for activity; it was slightly inhibiting in the presence of KCl alone but stimulated when NADPH was also present. Preincubation of the dialysed enzyme with DTT for up to 90 min did not increase its activity.

Effect of pH and kinetic constants

The pH optimum was 7 (Fig. 3). The K_m for CA1P was 0.43 mM and the measured $V_{max.}$ (Fig. 4) of 6.8 μmol/min per mg of protein suggests a specific activity less than that of other specific phosphatases in leaves. For example, phosphoglycollate phosphatase from spinach leaves (Husic & Tolbert, 1984) was purified to a specific activity of 75.7 μmol/min per mg of protein.

Specificity

Table 4 shows the phosphatase activity recorded with various phosphate esters. There was slight activity towards FBP, D-3-phosphoglycerate and inorganic pyrophosphate. Hydrolysis of RuBP was about 15% of the rate with CA1P. However, the 2-carboxy derivatives of both arabinitol 1,5-bisphosphate and ribitol 1,5-bisphosphate were hydrolysed more rapidly than CA1P. Only one phosphate group was released rapidly from the bisphosphates and it is presumed to have been that on C-1.

DISCUSSION

The CA1P phosphatase we describe from French bean has similarities with that purified from tobacco leaves by Salvucci & Holbrook (1989). Thus some of the effective purification steps, the pH optimum and the responses to NADPH and FBP are similar. The M_r values of the two enzymes are also similar. The phosphatase from tobacco leaves described by Gutteridge & Julien (1989), although reported not to be activated by NADPH, is likely to be the same enzyme as that described by Salvucci & Holbrook (1989) and dephosphorylates CABP and CRBP in addition to CA1P, like the enzyme from French-bean leaves (Table 3). The increases in activity of the tobacco enzyme (Salvucci & Holbrook, 1989) caused by KCl, K_2SO_4, $(NH_4)_2SO_4$ and NH_4Cl in the presence of NADPH are consistent with a response to ionic strength similar to that for the phosphatase from French bean (Table 2 and Fig. 2).

The salt concentrations needed for maximum activity (Table 2, Fig. 2) are not unnatural. Thus K^+ alone is present in chloroplasts at about 150 mM (Robinson, 1986) and the concentration of K^+ in the cytosol has been reported as being between 80 and 100 mM (Wyn Jones, 1981).

The inhibition of Rubisco activity at night is greater in French bean than in tobacco leaves. This may suggest higher concentrations of CA1P and a need for larger amounts of degrading enzymes to remove the inhibitor at dawn. Indeed the amounts of activity of the phosphatase (Table 1) recovered from French-bean leaves are larger than from tobacco leaves (Salvucci & Holbrook, 1989) and adequate to remove the CA1P. Assuming in Table 1 that half the soluble protein in the homogenate is Rubisco, and that at night all the catalytic sites (58 μmol; M_r of Rubisco 560000 and eight active sites per mol) would be occupied by CA1P, the phosphatase activity present is sufficient to degrade the CA1P in some 9 min. Salvucci & Holbrook (1989) quote a $t_{\frac{1}{2}}$ of 6.7 min for the degradation of CA1P *in vivo* following transfer of a plant, previously kept in the dark, directly into high irradiance; in nature the relief of inhibition of Rubisco in the morning is much slower than this.

Initially it was assumed that the mechanism of degradation of CA1P would be regulated by light, by reduction of the enzyme responsible by ferredoxin and thioredoxin. For this reason, DTT was used in buffers during extraction and purification of the phosphatase. However, the effects of DTT on CA1P phosphatase from French bean were variable; although it stimulated the activity of the phosphatase in crude extracts, it often slightly inhibited the purified enzyme unless BSA was also added. Holbrook *et al.* (1991) have provided convincing evidence for a loss of activity by the tobacco enzyme when it is dialysed to remove DTT; activity is partly restored when fresh DTT is added. We have dialysed the enzyme from French bean overnight against 50 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 2 mM-Ca^{2+} without loss of activity measured at an ionic strength

greater than 0.2. In Table 3, we show no significant effect of DTT on activity of dialysed pure enzyme. An activating effect of DTT would be indicative of light regulation of phosphatase by the ferredoxin/thioredoxin mechanism.

If the phosphatase is regulated by light, this is more likely to involve changes in concentrations of inorganic ions, metabolic intermediates and coenzymes than oxidation and reduction of the enzyme protein. In Table 3, we show that NADPH still stimulates the phosphatase even at increased ionic strength. Also FBP stimulates catalytic activity in the presence and absence of high salt. Holbrook *et al.* (1991) show that other phosphate esters produced during photosynthetic carbon assimilation, e.g. RuBP, phosphoglycollate and D-phosphoglycerate, also stimulate activity of the phosphatase of tobacco.

Activation by 10 mM-Mg^{2+} was observed at low ionic strength (Tables 2 and 3), but not in the presence of 0.2 M-KCl (Table 3). At concentrations lower than 10 mM, $MgCl_2$ (Table 2) inhibited the enzyme. Possibly there are beneficial effects of $MgCl_2$ through increasing ionic strength but also an adverse effect of Mg^{2+} on catalysis. Thus it seems unlikely that changes in Mg^{2+} concentration associated with the alkalization of the chloroplast stroma in the light can be a factor controlling CA1P phosphatase activity. The pH optimum of 7 (Fig. 3) is also not well matched to operation of the phosphatase in the chloroplast stroma in the light. Nevertheless, the phosphatase has been isolated from tobacco chloroplasts (Salvucci *et al.*, 1988; Gutteridge & Julien, 1989) and it has been assumed therefore that the enzyme is chloroplastic.

One aspect of the specificity of CA1P phosphatase is of special interest. Both the carboxy-D-pentitol bisphosphates that were available to us were better substrates than CA1P (Table 3). Thus a bisphosphate may be the main substrate in leaves. The activity with CRBP suggests that the second phosphate group is more important for enzyme binding than the steric arrangement about C-2.

Conclusions

French-bean leaves are a better source of CA1P phosphatase than tobacco leaves. The inclusion of the Dyematrex Green A column at an early stage considerably shortens the purification. Ionic strength has a marked effect on the catalytic activity of the enzyme and this needs to be taken into account in further studies especially in relation to regulation of activity. Since the greatest increase in activity occurs between values for ionic strength between 0.05 and 0.1, a range not much different from that in the usual buffered reaction mixtures used to measure enzyme rates, there is a danger of misinterpretation of the effects of ionic substances. However, the effects of NADPH and FBP are seen as specific and significant for regulation of the phosphatase activity *in vivo* since they are still observed in the presence of a high concentration of inorganic ions. The activity of the CA1P phosphatase is not dependent on the presence of DTT and it is unlikely therefore that the enzyme is regulated by the ferredoxin/thioredoxin system.

The award of a studentship from the Lawes Agricultural Trust is gratefully acknowledged by A. H. K-S.

REFERENCES

Ames, B. N. (1966) Methods Enzymol. **8**, 115–116
Beck, E., Stransky, H. & Furbringer, M. (1971) FEBS Lett. **13**, 229–234
Beck, E., Scheibe, R. & Reiner, S. (1989) Plant Physiol. **90**, 13–16
Berry, J. A., Lorimer, G. H., Pierce, J., Seemann, J. R., Meek, J. & Freas, S. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 734–738
Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
Gutteridge, S. & Julien, B. (1989) FEBS Lett. **254**, 225–230
Gutteridge, S., Parry, M. A. J., Burton, S., Keys, A. J., Mudd, A., Feeney, J., Servaites, J. C. & Pierce, J. (1986) Nature (London) **324**, 274–276
Gutteridge, S., Reddy, G. S. & Lorimer, G. H. (1989) Biochem. J. **260**, 711–716
Holbrook, G. P., Bowes, G. & Salvucci, M. E. (1989) Plant Physiol. **90**, 673–678
Holbrook, G. P., Galasinski, S. C. & Salvucci, M. E. (1991) Plant Physiol. **97**, 894–899
Husic, H. D. & Tolbert, N. E. (1984) Arch. Biochem. Biophys. **229**, 64–74
Keys, A. J., Kingston-Smith, A. H. & Parry, M. A. J. (1989) in Current Research in Photosynthesis III (Baltsheffsky, M., ed.), pp. 395–398, Kluwer Academic Publishers, The Netherlands
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Pierce, J., Tolbert, N. E. & Barker, R. (1980). Biochemistry **19**, 934–962
Robinson, S. P. (1986) Photosynth. Res. **10**, 93–100
Salvucci, M. E. & Holbrook, G. P. (1989) Plant Physiol. **90**, 679–685
Salvucci, M. E., Holbrook, G. P., Anderson, J. C. & Bowes, G. (1988) FEBS Lett. **231**, 197–201
Seemann, J. R., Kobza, J. & Moore, B. D. (1990) Photosynth. Res. **23**, 119–130
Van Veldhoven, P. P. & Mannaerts, G. P. (1987) Anal. Biochem. **161**, 45–48
Vu, C. V., Allen, L. H. & Bowes, G. (1983) Plant Physiol. **73**, 729–734
Wong, C-H., McCurry, S. D. & Whitesides, G. M. (1980) J. Am. Chem. Soc. **102**, 7938–7939
Wyn Jones, R. G. (1981). in Physiological Processes Limiting Plant Productivity (Johnson, C. B., ed)., pp. 271–292, Butterworths, London

Received 2 March 1992/30 April 1992; accepted 12 May 1992