

Isolation and Characterization of Two Enzymes Capable of Hydrolyzing Fructose-1,6-Bisphosphatase from the Lichen *Peltigera rufescens*¹

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ABSTRACT

Two enzymes capable of hydrolyzing fructose-1,6-bisphosphate (FBP) have been isolated from the foliose lichen *Peltigera rufescens* (Weis) Mudd. These enzymes can be separated using Sephadex G-100 and DEAE Sephacel chromatography. One enzyme has a pH optimum of 6.5, and a substrate affinity of 228 micromolar FBP. This enzyme does not require MgCl₂ for activity, and is inhibited by AMP. The second enzyme has a pH optimum of 9.0, with no activity below pH 7.5. This enzyme responds sigmoidally to Mg²⁺, with half-saturation concentration of 2.0 millimolar MgCl₂, and demonstrates hyperbolic kinetics for FBP ($K_m = 39$ micromolar). This enzyme is activated by 20 millimolar dithiothreitol, is inhibited by AMP, but is not affected by fructose-2,6-bisphosphate. It is hypothesized that the latter enzyme is involved in the photosynthetic process, while the former enzyme is a nonspecific acid phosphatase.

FBPase² (EC 3.1.3.11) is an important enzyme of carbon metabolism, involved in gluconeogenesis, the pentose phosphate cycle and photosynthesis. As a result, three forms of this enzyme are usually present in most plants (15, 17, 28), distinguishable by their compartmentation, regulatory properties and their response to differing pH and ionic environments. One FBPase acts as a Mg²⁺-independent acid phosphatase (17, 20), while the other two are Mg²⁺-dependant alkaline phosphatases. Of the latter two, the cytosolic FBPase is inhibited by AMP, F-2,6-P, and high concentrations of FBP (8, 13, 30). The photosynthetic enzyme is not inhibited by these compounds, but it is activated by thioredoxin or DTT (4, 5, 23, 29).

The photosynthetic FBPase is an important regulatory enzyme in the Calvin-Benson cycle. Studies on the changes in metabolite levels during light/dark shifts indicate that the hysteretic activation of this enzyme is responsible for the lag in CO₂ fixation observed at the onset of the light period (14). In addition, it has been suggested that the FBPase may also be a rate-limiting step during steady state photosynthesis (14).

Brown and Kershaw (2) have recently characterized the seasonal photosynthetic pattern in the foliose lichen *Peltigera rufescens*. They observed a large shift in the temperature optimum of CO₂ fixation throughout the year, and postulated that this re-

sulted from a change in a rate-limiting step of the Calvin-Benson cycle, controlled by the activity of either FBPase or ribulose bisP carboxylase.

Neither of these enzymes have been previously studied in a lichen, and this paper reports on the separation and characterization of the two enzymes capable of hydrolyzing FBP that have been isolated from *P. rufescens*, with the specific aim of isolating the photosynthetic enzyme. An accompanying paper (3) reports on the seasonal changes observed for this FBPase and relates this enzyme to photosynthetic temperature acclimation.

MATERIALS AND METHODS

Material. *Peltigera rufescens* (Weis) Mudd. Was collected from rock outcrops in the Port Severn area of Central Ontario, transported to the laboratories at McMaster University, and then maintained in an air-dry state in growth chambers during the experimental period (for details, see Brown and Kershaw [3]).

Protein Extraction. Forty to 60 g (air-dry weight) of fresh, nonfruiting lobes were carefully dissected from the lichen mat and soaked overnight (16 h) in distilled H₂O. Extraction buffer (30 mM Pipes, 20 mM 2-mercaptoethanol, and 20 mM sodium metabisulfite, adjusted to pH 6.6) (10 ml·g⁻¹ dry weight) and insoluble PVP (0.5 g·g⁻¹ dry weight) were added to the drained thalli and this was ground with a mortar and pestle. The slurry was further processed for 20 min with a motorized tissue homogenizer. The resulting suspension was sonicated for a total of 20 min, and then centrifuged in a swinging bucket rotor on a Beckman TJ 6 refrigerated centrifuge for 20 min at 1500g. The supernatant was reserved and the pellet was resuspended in approximately 300 ml of the extraction buffer, then resonicated and again centrifuged. The supernatants were combined and the final pellet discarded.

FBPase Purification. The enzyme was purified using a modification of the method of Buchanan *et al.* (4). The crude extract was acidified to pH 4.5 by the addition of 1.0 M acetic acid. The precipitate formed was collected by centrifugation (1500g for 30 min) and resuspended in 150 ml of the extraction buffer. (NH₄)₂SO₄ was added to 40% of saturation. This solution was centrifuged in a Sorval RC 2B centrifuge at 20,000g for 15 min. The pellet was discarded and the supernatant was brought up to 80% of saturation with additional (NH₄)₂SO₄. This was centrifuged as above, the supernatant was discarded, and the pellet was resuspended in 15 ml of extraction buffer. This was chromatographed on a 75 × 2.5 cm column of Sephadex G-100 (previously equilibrated with the same extraction buffer) at a flow rate of 18 ml·h⁻¹. Fractions high in FBPase activity were combined and loaded onto a 10 × 1.6 cm column of DEAE Sephacel equilibrated with the extraction buffer. A 0.0 to 0.3 M NaCl gradient was produced in 300 ml of extraction buffer and used to elute the protein from the column at a flow rate of 18 ml·h⁻¹. Fractions

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² Abbreviations: FBPase, fructose-1,6-bisphosphatase; S_{0.5}, half-saturation concentration; FBP, fructose-1,6-bisphosphate; F-2,6-P, fructose-2,6-bisphosphate.

containing FBPase activity were combined and concentrated by ultrafiltration through an Amicon YM 30 membrane.

Activity of the purified FBPase declined rapidly over a storage period of 2 weeks at 4°C. However, the addition of BSA to a concentration of 5% and storage at -15°C was sufficient to maintain high activity for several months.

FBPase Assay. Enzyme activity was assayed using a modification of the spectrophotometric assay of Racker and Schroeder (20). The formation of fructose 6-P was linked to an NADPH-generating system by phosphoglucose isomerase and glucose 6-P dehydrogenase and was monitored continuously at 340 nm in a temperature regulated Varian DMS-100 spectrophotometer. The complete assay included 3.0 mM FBP, 1.0 mM NADP, 10 mM MgCl₂, 20 mM DTT, 4 units of P-glucose isomerase, and 4 units of glucose 6-P dehydrogenase in 1.0 ml of either 100 mM Pipes (pH 6.6) (Fraction A enzyme) or 100 mM Tris-HCl (pH 8.5) (Fraction B enzyme). The assay was started with the addition of sufficient FBPase to produce a change in *A* of 0.005 to 0.01 units·min⁻¹. All assays were done at 25°C.

Kinetic Parameter Calculations. *K_m* and *V_{max}* values were calculated using the direct linear plot (6). Initial rates were determined by the cord method of Waley (27). For the pH relationships, Pipes buffer was used from pH 5.5 to 7.0, while Tris-HCl was used from pH 7.5 to 10.0. Protein content was measured with the Coomassie blue assay (Biorad Laboratories) using BSA as a standard.

RESULTS

FBPase activity in the crude extract from *Peltigera rufescens* demonstrated a broad response to pH (Fig. 1), with local pH

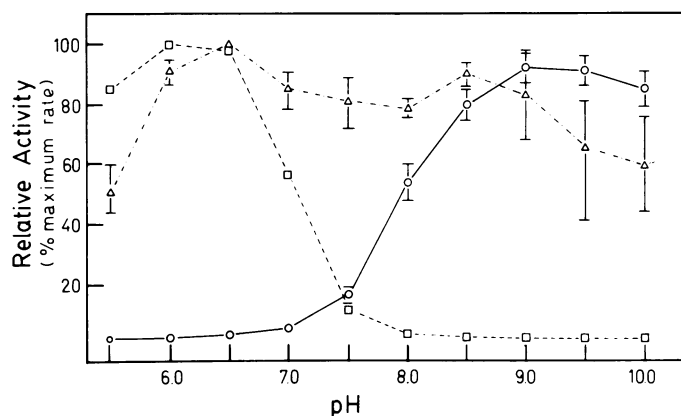


FIG. 1. The relationship between pH and FBPase activity of the crude extract (Δ - Δ), fraction A (\square - \square) and fraction B (\circ - \circ) enzymes.

optima at pH 6.5 and pH 8.5. Enzyme activity at pH 8.5 was MgCl₂ dependent, while that at pH 6.5 did not require Mg²⁺, suggesting that several enzymes capable of hydrolyzing FBP were present in the extract.

Acidification of the crude extract to pH 4.5 partially separated these fractions, with the activity at pH 8.5 concentrated in the pellet (Table I).

The pH 4.5 pellet was further fractionated with (NH₄)₂SO₄, and then chromatographed in a Sephadex G-100 column. FBPase activity eluted in two peaks following the void volume (Fig. 2). The leading peak was high in pH 8.5 activity while the trailing peak contained the majority of the pH 6.5 activity. Fractions of the latter peak were combined and designated as fraction A enzyme. Fractions in the leading peak were combined and further purified on a DEAE Sephacel column (Fig. 3). Again two peaks of FBPase activity were observed. Fractions enriched in pH 8.5 activity eluted prior to a NaCl concentration of 0.25 M (fraction B enzyme), while a small amount of pH 6.5 activity eluted at 0.28 M NaCl.

Isolation of the fraction A enzyme resulted in an 8-fold purification, with a specific activity of 0.43 $\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ (Table I). The fraction B enzyme was purified to a much greater extent (60-fold, Table I), with specific activities as high as 8.3 $\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ achieved in some isolations (Table III; Ref. 3).

The pH profile of these enzymes are shown in Figure 1. Fraction A has optimal activity at a pH of 6.0, with little activity above pH 7.5. In direct contrast, fraction B is active at alkaline pH, with an optimum at pH 9.0 and no activity below pH 7.5.

The activity of the fraction B enzyme is dependent upon Mg²⁺ (Fig. 4). The response of this enzyme is sigmoidal, with an apparent *S*_{0.5} of 2.0 mM MgCl₂, and a Hill coefficient of 1.4. Activity of the fraction A enzyme is unaffected by the Mg²⁺ concentration of the assay.

Both fraction A and B enzymes show a hyperbolic relationship to substrate concentration (Fig. 5), with a *K_m* of 228 \pm 28 (mean \pm SE) and 39 \pm 9 μM , respectively. Neither enzyme is inhibited at high (3.0 mM) substrate concentrations.

A study of the regulatory properties of the two fractions indicate that AMP inhibits the activity of both enzymes (Table II). Fraction B was the more sensitive enzyme, with a 56% inhibition at 0.2 mM AMP, in contrast to only 20% inhibition of the fraction A enzyme at this concentration.

F-2,6-P has been recently shown to act as a competitive inhibitor of the cytosolic FBPase (8, 13, 17). Table III shows the effect of 2 μM F-2,6-P on the activity of the fraction B enzyme. There is no significant inhibition of enzyme activity at any of three FBP concentrations tested (0.03, 0.3, 3.0 mM). However, there was a slight synergistic effect between F-2,6-P and AMP for two of the four extracts tested (Table III).

Table I. Purification of the Fraction A and B FBPases from *P. rufescens*

Fraction	Volume <i>ml</i>	Total Protein <i>mg</i>	FBPase Activity			
			pH 8.5		pH 6.5	
			Specific <i>nmol}\cdot\mu\text{g}^{-1}\cdot\text{min}^{-1}</i>	Total $\mu\text{mol}\cdot\text{min}^{-1}$	Specific <i>nmol}\cdot\mu\text{g}^{-1}\cdot\text{min}^{-1}</i>	Total <i>nmol}\cdot\text{min}^{-1}</i>
Crude extract	1260	1294	0.031	40	0.056	72.6
pH 4.5 pellet	300	697	0.066	46	0.021	14.1
40-80% (NH ₄) ₂ SO ₄	15.5	88	^a	^a	^a	^a
G-100						
Fraction A	3.3	3.7	0.012	0.004	0.423	0.018
Fraction B	43	24	0.55	13.6	0.019	0.47
DEAE sephacel						
Fraction B	20	1.0	1.91	1.92	0.036	0.04

^a FBPase activity was strongly inhibited by (NH₄)₂SO₄, therefore activity could not be determined at this step.

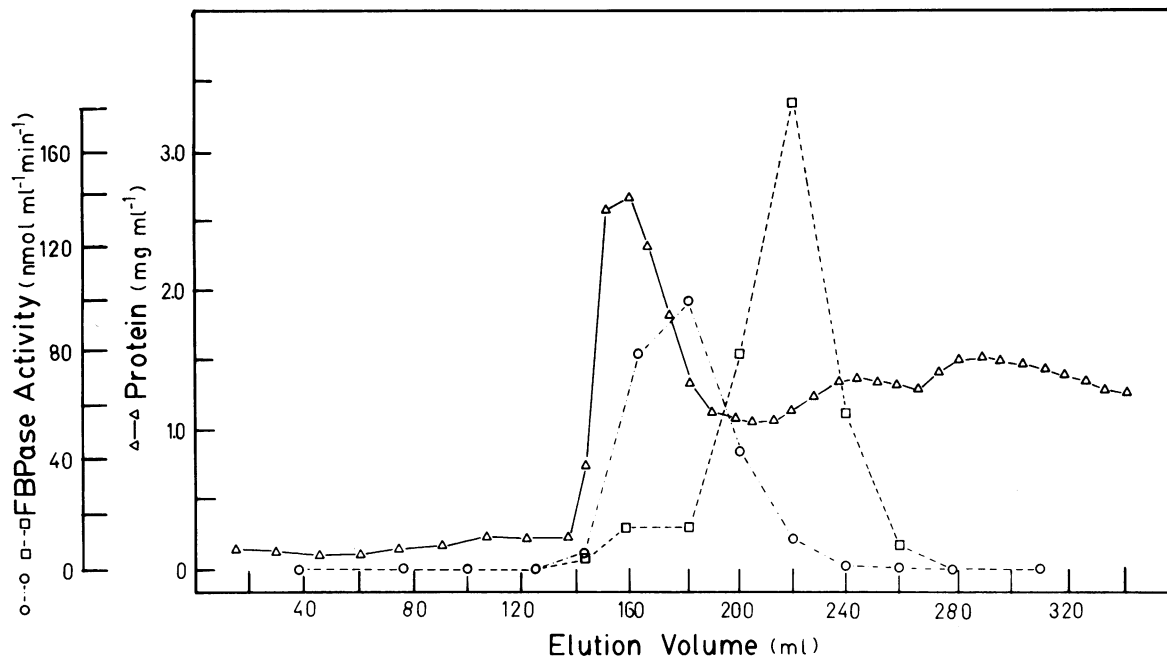


FIG. 2. The elution pattern of FBPase activity by a Sephadex G-100 column. Enzyme activity was assayed at pH 6.5 (\square - \square) and pH 8.5 (\circ - \circ). Protein concentrations (Δ - Δ) were calculated from the A at 280 and 260 nm using the equation: $\mu\text{g} \cdot \text{ml}^{-1} \text{ protein} = 1.55 A_{280\text{nm}} - 0.76 A_{260\text{nm}}$ (5).

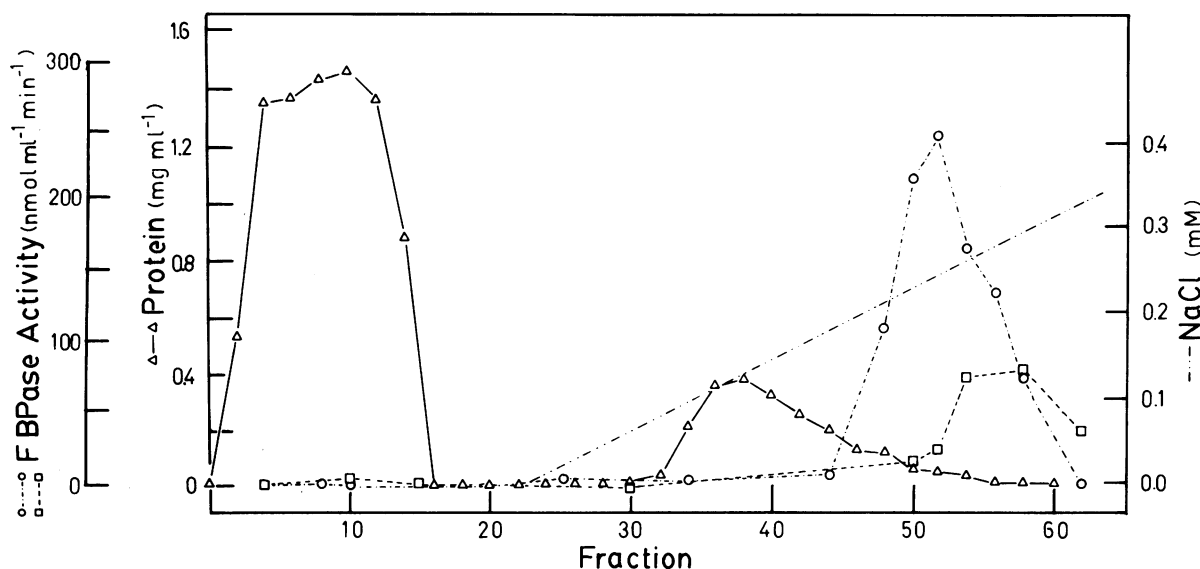


FIG. 3. The pattern of FBPase activity eluted from a DEAE Sephacel column by a NaCl gradient (---). Enzyme activity was assayed at pH 6.5 (\square - \square) and pH 8.5 (\circ - \circ). Protein concentrations (Δ - Δ) were calculated as in Figure 2.

These enzymes also differ considerably in their time course of activity. Enzyme activity in fraction A is constant with time, whereas a 15 to 20 min lag period exists before maximal rates are observed in the fraction B enzyme (Fig. 6). This lag could not be eliminated by a 24 h preincubation with 20 mM DTT (Fig. 7) (29). However, the omission of DTT from the assay significantly reduces the maximum activity of the enzyme (Table IV; Fig. 6). DTT had no effect on the substrate affinity or the length of the lag period.

DISCUSSION

Evidence obtained from the hydrolysis of FBP in the crude extracts indicated the presence of several phosphatases in *Peltigera rufescens* (Fig. 1). These have been purified into two components. Fraction A is a Mg^{2+} -independent acid phosphatase

while fraction B is a Mg^{2+} -dependent alkaline phosphatase (Figs. 1 and 4).

Three different FBPases have been characterized in higher plants. A nonspecific acid phosphatase capable of hydrolyzing FBP has been isolated from the leaves of spinach and soybeans (20), as well as corn and pea roots (28). This enzyme has an acidic pH optimum and shows no response to MgCl_2 . The fraction A enzyme from *P. rufescens* is similar to this enzyme. It is active below pH 7.5 (Fig. 1), and does not require MgCl_2 (Fig. 4). Although the fraction A enzyme was not tested for substrate specificity, the high K_m observed for FBP (228 μM) suggests it may not be specifically involved in FBP metabolism.

The photosynthetic enzyme has been shown to exist in several forms. The enzyme undergoes a pH-dependent conversion between an inactive dimer and an inactive tetramer (20, 29). The *in vivo* activation of this tetramer involves the reduction of two

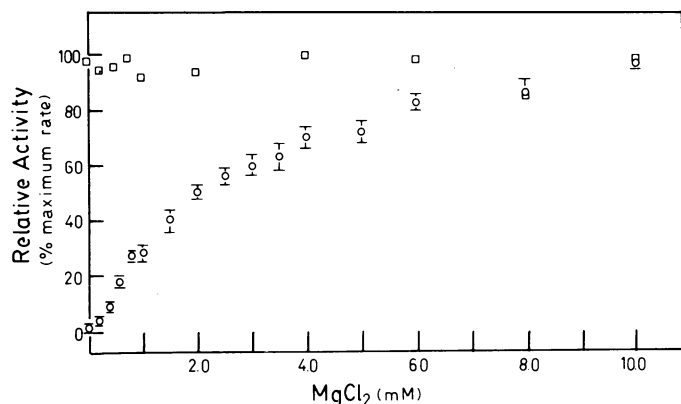


FIG. 4. The effect of $MgCl_2$ concentration on the activity of the purified fraction A (\square , assayed at pH 6.5) and fraction B (\circ , assayed at pH 8.5) enzymes.

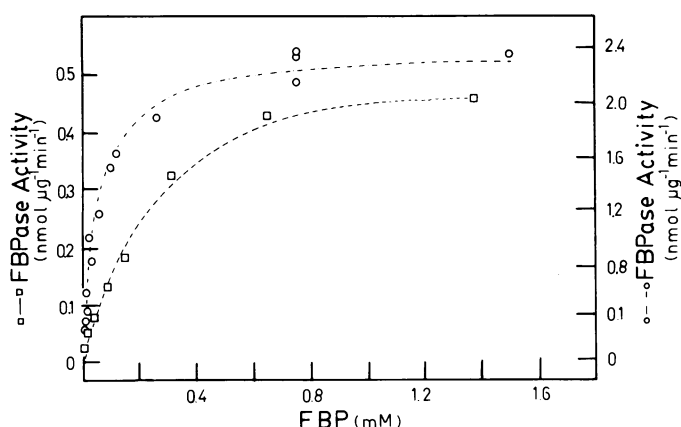


FIG. 5. The effect of FBP concentration on the activity of the purified fraction A (\square , assayed at pH 6.5) and fraction B (\circ , assayed at pH 8.5) enzymes.

Table II. Effect of AMP on FBPase Activity

Fraction	Concentration of AMP	Inhibition mean \pm SE
	mm	%
A	0.2	20
	2.0	61 \pm 1
B	0.2	56 \pm 7
	2.0	71 \pm 2.6

disulfide bridges via a ferredoxin-thioredoxin activation system (18, 23). This process can be mimicked *in vitro* using DTT as the reductant (21, 29). However, this is a slow process, with lag periods lasting up to 20 min. The activated enzyme isolated from spinach chloroplasts has a pH optimum above pH 8.0, with no activity observed below pH 7.5 (15, 16, 29). This enzyme responds sigmoidally to Mg^{2+} concentration, with a $S_{0.5}$ of 1.0 to 3.0 mM $MgCl_2$ (4, 5, 18). It is uncertain if the response of the photosynthetic enzyme to substrate concentration is sigmoidal (18, 19, 29) or hyperbolic (5), but half-maximal velocity is achieved at concentrations in the range of 30 to 80 μM FBP.

The third enzyme, a cytosolic FBPase isolated from the leaves of spinach, is thought to be involved in glucose metabolism. This enzyme has a much higher affinity for both FBP (K_m of 3–25 μM) and $MgCl_2$ ($S_{0.5}$ of 0.1–1.0 mM) than either the photosyn-

Table III. Inhibitory Effects of 2 μM F-2,6-P and 0.2 mM AMP on the Activity of the Fraction B FBPase from Four Separate Purifications

Extraction	Inhibitor	FBP Concentration (mm)		
		3.0	0.3	0.03
$\mu M \cdot mg^{-1} \cdot min^{-1}$				
1	Control	8.30 \pm 0.36	8.57 \pm 0.12	5.68 \pm 0.22
	AMP	2.86 \pm 0.27		
	F-2,6-P	7.43 \pm 0.33	7.76 \pm 0.19	6.32 \pm 0.21
	F-2,6-P + AMP	1.75 \pm 0.14 ^a		
2	Control	3.62 \pm 0.09		
	AMP	3.35 \pm 0.18		
	F-2,6-P	3.22 \pm 0.21		
	F-2,6-P + AMP	3.20 \pm 0.07		
3	Control	2.80 \pm 0.05		
	AMP	1.04 \pm 0.04		
	F-2,6-P	2.63 \pm 0.04 ^b		
	F-2,6-P + AMP	0.73 \pm 0.02 ^a		
4	Control	2.98 \pm 0.21	4.21 \pm 0.11	2.18 \pm 0.03
	AMP	2.86 \pm 0.21		
	F-2,6-P	2.94 \pm 0.19	3.93 \pm 0.19	2.02 \pm 0.03 ^b
	F-2,6-P + AMP	2.26 \pm 0.01		

^a Significantly less than inhibition by AMP alone, $P > 0.05$. ^b Significantly less than control, $P > 0.05$.

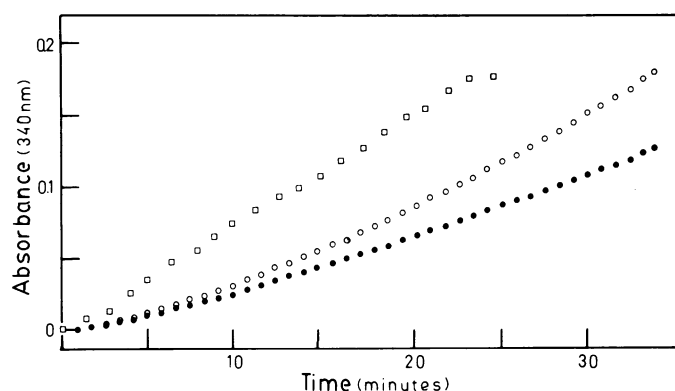


FIG. 6. The effect of DTT concentration on the FBPase activity. The change in absorbance with time was monitored at a FBP concentration of 0.313 mM in an assay containing either 0.0 mM DTT (closed) or 20 mM DTT (open) at pH 6.5 (\square , fraction A) or pH 8.5 (\circ , \bullet , fraction B).

thetic (8, 13, 15, 24, 30) or the fraction B FBPase. The activity of the cytosolic enzyme is optimal around pH 7.5 to 8.0, and declines rapidly as pH drops below 7.0 (15, 30). Unlike the photosynthetic enzyme, it does not undergo DTT activation (15), and it is strongly inhibited by AMP, F-2,6-P and substrate concentrations higher than 0.1 mM FBP (8, 13, 17, 24).

The fraction B enzyme isolated from *P. rufescens* is most similar to the photosynthetic enzyme. Its activity is totally alkaline (Fig. 1), its substrate affinity (39 μM) and requirement for $MgCl_2$ ($S_{0.5}$ = 2.0 mM) is within the range reported for this enzyme, and it is activated by DTT (Fig. 6; Table IV). Pretreatment with DTT, however, does not decrease the lag period nor increase the enzyme's substrate affinity, as has been observed with the photosynthetic enzymes of higher plants (5, 23, 29). The lack of inhibition by F-2,6-P and high concentrations of FBP (Table III) is evidence that this enzyme is not cytosolic in origin or function. However, the inhibition by AMP (Tables II and III) is more characteristic of the cytosolic, and not the photosynthetic FBPase (4, 25).

However, the phycobiont of *P. rufescens* is a cyanobacterium

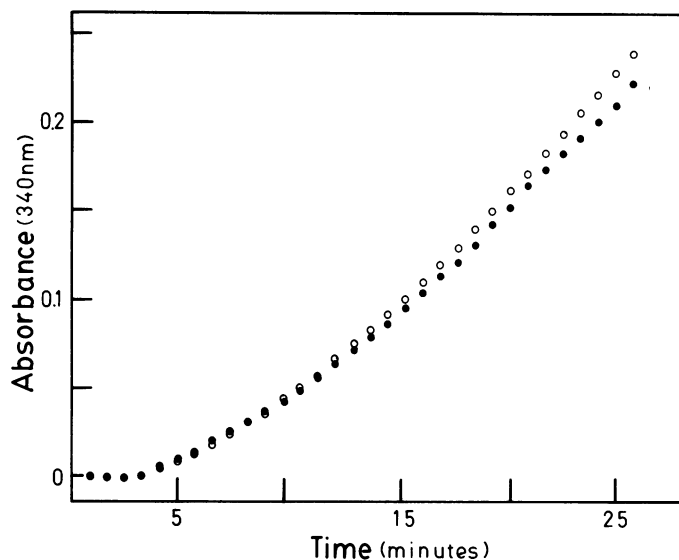


FIG. 7. The effects of a DTT pretreatment on the activity of the fraction B enzyme. The change in absorbance with time of the enzyme pretreated with 20 mM DTT for either 24 h (●) or 0 h (○) was assayed in the presence of 20 mM DTT at pH 8.5.

Table IV. Effects of DTT on the Michaelis-Menton Parameters of the Fraction B FBPase from Three Separate Purifications

Extraction	Parameter	Assay Condition ^a		t-test
		20 mM DTT	No DTT	
1	K_m (μM)	43 ± 5	45 ± 4	0.299
	V_{max} ^b	10.28 ± 0.82	2.78 ± 0.26	8.68 ^c
	n	5	5	
2	K_m (μM)	58 ± 6	45 ± 9	1.6
	V_{max} ^b	5.21 ± 0.49	2.29 ± 0.17	5.67 ^c
	n	4	4	
3	K_m (μM)	33 ± 5	49 ± 3	2.54 ^d
	V_{max} ^b	2.39 ± 0.13	0.93 ± 0.14	7.79 ^c
	n	5	4	

^a Mean \pm SE. ^b nmol \cdot μg^{-1} min⁻¹. ^c P > 0.01. ^d P > 0.05.

(*Nostoc*). In contrast to the spinach photosynthetic enzyme, but closely corresponding with the fraction B enzyme, FBPase activity for *Anacystis* and *Synechococcus* is sensitive to AMP concentrations in the 0.2 to 2.0 mM range (1, 11, 25). The FBPases isolated from these cyanobacteria appear to be similar to the photosynthetic enzyme in other respects (1, 22, 25). Activity is restricted to an alkaline pH, with an optimum at pH 8.0. Bishop (1) reported hyperbolic kinetics for both FBP and Mg^{2+} with K_m of 88 and 0.95 mM, respectively. Udvardy *et al.* (25) report a similar K_m of 60 μM for FBP; however, they observed a sigmoidal curve with MgCl_2 , with a $S_{0.5}$ of 5 mM. The specific activities of the purified FBPases from these studies (0.88 (22), 2.81 (1), and 8.1 $\mu\text{mol} \cdot \text{mg}^{-1} \text{min}^{-1}$ (25) are similar to the fraction B enzyme (Tables I, III, IV).

More recent studies of *Synechococcus leopoliensis* have shown two electrophoretically distinct FBPases in this cyanobacterium (11, 12). The most prevalent form was shown to undergo DTT activation and a dimer-tetramer interconversion similar to the spinach photosynthetic enzyme (12). This form, which was purified to a much higher degree than the fraction B enzyme (specific activity of 37.4 $\mu\text{mol} \cdot \text{mg}^{-1} \text{min}^{-1}$) is also sensitive to AMP inhibition (11).

It becomes apparent that the fraction B enzyme is most similar

to photosynthetic FBPase isolated from cyanobacteria and similarly may be involved in the photosynthetic carbon metabolism in *P. rufescens*. This could be verified by studying the FBPase extracted from the cyanobacterium isolated from the lichen. Unfortunately, this has not been possible since a sufficient quantity of *Nostoc*, free of fungal contaminants, could not be obtained.

The lack of a cytosolic FBPase is also somewhat puzzling. It is possible that the fraction B enzyme serves a dual role in the phycobiont, as suggested for *Anacystis* (1), but this would not explain the apparent lack of a cytosolic fungal FBPase. The FBPases isolated from the yeasts *Saccharomyces cerevisiae* and *Candida utilis* (10) appear to be similar to the spinach cytosolic enzyme, and are thus different from either the fraction A or B enzymes. It is more likely that the fungal cytosolic enzyme was lost during the purification of the fraction A and B enzymes. The crude extract had a high enzyme activity at pH 7.5 which was not observed in either of the purified enzymes (Fig. 1), adding support to this theory.

Although seasonal changes in photosynthetic capacity mediated by enzymes are of limited occurrence in lichens, they appear to be particularly important in some circumstances. Coxson and Kershaw (7) have observed a seasonal increase in photosynthetic capacity at low temperatures for *Caloplaca trachyphylla*, an adaptation to winter chinook sequences. In addition, Brown and Kershaw (2) have recorded a seasonal change in the temperature optimum for this process in *P. rufescens*. Enzymic studies in lichens have been hindered due to their resistance to mechanical disruption, low protein yields, and high content of secondary compounds (9, 26). The successful isolation of a photosynthetic FBPase from *P. rufescens* has now enabled a more in depth study of the photosynthetic capacity changes observed in this species (3).

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