ABSTRACT
ADP-glucose pyrophosphorylase (AGPase) is a key regulatory enzyme in glycogen synthesis in bacteria and starch synthesis in higher plants. The enzyme uses glucose 1-phosphate (G1P) and ATP as substrates to produce ADP-glucose (the activated form of glucose for the next biosynthetic enzyme) and inorganic pyrophosphate (PPi) (Fig. 1). In general, the enzymatic activity assay of this reaction is performed by measuring the amount of 14C-labeled ADP-glucose produced from 14C-labeled G1P and ATP. This method, however, has the disadvantage of working with radioactive material and its accompanying restrictive regulations in usage and record keeping. Moreover, it is relatively time consuming. Alternatively, the assay can be performed enzymatically by measuring the amount of PPi produced by AGPase. This can be accomplished using PPi-dependent phosphofructokinase (PP-PFK), aldolase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase (NADP+) (Fig. 2) and then measuring the conversion of NADPH to NADP+ by measuring the decrease in absorbance at 340 nm. To perform the spectrophotometric assay it is essential to obtain purified PP-PFK, the only enzyme in this scheme which is not available commercially. Therefore, I purified PP-PFK from Borrelia burgdorferi by expressing it in Escherichia coli BL21 cells and applying a two-step purification protocol. First, the bacterial crude extracts were treated with polyethyleneimine to remove DNA followed by Sephadex G-25 size exclusion chromatography. The nucleic acid free extracts were then fractionated on cellulose phosphate bi-functional cation exchange chromatography. Using this protocol I was able to purify the protein to near homogeneity and assess its kinetic properties.

INTRODUCTION
Starch is a main reserve stored in many harvestable edible sink organs of plants and, hence, a major constituent of the human diet. Understanding the biosynthesis of starch can help identify ways to increase starch yield and, in turn, increase overall plant productivity and crop yield. The process depends on source-sink relationships. Source-sink relationship can be described as the extent to which a plant can photosynthesize and fix CO2 through source leaves, and the extent to which the fixed carbon can then be assimilated by sink tissues to form reserves such as starch (1). Enzymes involved in the starch metabolism can be engineered to obtain a higher yield of starch production. ADP-glucose pyrophosphorylase (AGPase) is an important enzyme that catalyzes the rate-limiting step of starch biosynthesis (2). When AGPase is available, it catalyzes the conversion of glucose 1-phosphate (G1P) and ATP into ADP-glucose (AGP) and inorganic pyrophosphate (PPi). Assays of the reaction are normally monitored with radiolabeled 14C-G1P. In order to continue our studies without using radioisotopes, we have developed an alternative assay through coupling additional enzyme reactions (Fig. 2). To proceed, it was essential to purify PPi-dependent phosphofructokinase (PP-PFK), the only enzyme commercially available. TABLE 1 shows the overall purification process where thirty-six percent of PP-PFK is purified and recovered from crude extract.

METHODS
Expression and purification of recombinant PP-PFK.
To purify PP-PFK the methods used were in accordance with Deng et al (3). The plasmid encoding the enzyme was first transformed into E. coli BL21 (DE3) cells, plated onto ampicillin (200 μg/ml) media and incubated overnight. Three colonies were then incubated into 25 ml of LB media and grown at 37°C. The seed culture was then transferred into one-liter of NCTC-109 media (10 g NZ-ammonite, 3 g yeast extract, 5 g NaCl, 2 g MgSO4 • 7 H2O and 1 g casamino acids, pH 7.0) and incubated at room temperature until optical density (OD600) reached ~0.7. The cells were then induced with a final concentration of 0.1 mM IPTG for twenty hours. The cells were centrifuged and resuspended in 25 ml of binding buffer (20 mM Pipes pH 7.0, 0.1 mM EDTA, 1 mM DTT). The cells were then disrupted by sonication and the cell debris pelleted by centrifugation at 20,853g for 20 minutes. The supernatant fluid was collected and 10% polyethyleneimine (PEI) was slowly added to a final concentration of 0.1% and mixed for ten minutes. The precipitated nucleic acids were then removed by centrifugation at 10,000g for 10 minutes. The resulting supernatant was then passed through Sephadex G-25 column (15 cm × 2.5 cm) to remove excess PEI. The fractions were run on a 12% polyacrylamide gel, and fractions containing the enzyme were combined. These combined fractions were then passed through a phosphocellulose (P1, Whatman) column (5 cm × 2.5 cm). After extensive washing, the enzyme was eluted with binding buffer containing 0.5 mM fructose 1,6 biphosphate. Fractions from both columns were collected by monitoring absorbance at 340 nm. The precipitate was stored at -20°C. The enzyme was then utilized to test the alternative assay performed in the absence of radioisotopes.

PP-PFK assay.
All activity assays of PP-PFK were performed at room temperature using spectrophotometric assays to measure the decrease in absorbance at 340 nm until no further changes in absorbance were observed. Assays were performed in 100 μl of solution containing 20 mM potassium phosphate buffer (pH 7.2), 1 mM EDTA, 5 mM MgCl2, 2 mM F6P, 0.2 mM NADH, and 0.25 units each of aldolase, triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase.

AGPase Assay
AGPase was assayed in a 200 μl solution of 100 mM Heps (pH 7.0), 3 mM DTT, 10 mM MgCl2, 2 mM ATP, 5 mM 3-PGA, 2 mM G1P and 0.4 mg/ml BSA. Assays were initiated by addition of enzyme and performed at 37°C for 2.5 minutes. Reactions were then stopped by boiling the samples for one minute. The amount of PPi produced by AGPase was determined from a standard curve (Plotted by different concentrations of NaPPi and omitting the enzyme) and measured spectrophotometrically at 340 nm by mixing 100 μl of the first reaction and 200 μl of the developing mixture. The developing mixture contains final concentrations of 25 mM imidazole (pH 7.2), 1 mM EDTA, 5 mM MgCl2, 0.2 mM NADH, and 0.25 units each of aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and 0.8 μg of the purified PP-PFK. Both solutions were prepared.

CONCLUSIONS
Using a combination of PEI precipitation and, Sephadex G-25 and P11 column chromatography steps, PP-PFK was successfully purified to near homogeneity. In FIGURE 3A a single polypeptide band corresponding to PP-PFK can be seen clearly at 62 kDa in fractions eight through eleven. PP-PFK was used in preliminary tests for an alternative assay not requiring the use of radioactive substrates. FIGURE 4 shows a direct comparison of the enzyme activities measured by radioactive and non-radioactive assays. Non-radioactive assay exhibits reliability, and the assay will continue to be optimized. The enzyme can be utilized on additional mutants in the alternate assay.

REFERENCES

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