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Purification of phosphinothricin acetyltransferase using Reactive brown 10 affinity in a single chromatography step

Cunxi Wang *, Thomas C. Lee, Kathleen S. Crowley, Erin Bell

Monsanto Company, 800 North Lindbergh Blvd, St. Louis, MO 63167, United States

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ABSTRACT

The expression of phosphinothricin *N*-acetyltransferase (PAT) protein in transgenic plants confers tolerance to the herbicide glufosinate. To enable the characterization of PAT protein expressed in plants, it is necessary to obtain high purity PAT protein from the transgenic grain. Because transgenically expressed proteins are typical present at very low levels (i.e. 0.1–50 μg protein/g grain), a highly specific and efficient purification protocol is required to purify them. Based on the physicochemical properties of PAT, we developed a novel purification method that is simple, time-saving, inexpensive and reproducible. The novel method employs a single chromatography step using a reactive dye resin, Reactive brown 10-agarose. Reactive brown 10 preferentially binds the PAT protein, which can then be specifically released by one of its substrates, acetyl-CoA. Using Reactive brown 10-agarose, PAT protein was purified to homogeneity from cottonseed with high recovery efficiency. As expected, the Reactive brown 10-produced PAT was enzymatically active. Other applications of the method on protein expression and purification, and development of PAT enzymatic inhibitors were also discussed.

Introduction

Phosphinothricin *N*-acetyltransferase (PAT)[1] is an enzyme that acetylates the free NH_2 group of L-phosphinothricin (L-PPT) in the presence of acetyl-CoA as a co-substrate [1,2]. It is highly specific for L-PPT and does not acetylate other L-amino acids or structurally similar molecules [2]. L-PPT is a glutamate analog that can inhibit glutamine synthetase activity in plants, resulting in the accumulation of ammonia to toxic levels and impairment of photosynthesis [3–5]. Thus PPT, the ammonium salt of which is also known as glufosinate, is a broad-spectrum herbicide [1,2,5]. The introduction of a PAT gene into a plant genome can confer resistance to glufosinate herbicide during post-emergent applications [1,2,6,7]. Commercially available LibertyLink® products provide glufosinate-tolerance in several crops including cotton, corn, soybean and canola.

To assess the structural and functional characteristics of PAT in a new transgenic crop under development, it is necessary to isolate PAT from the seed of that product. Previously described methods for PAT purification involve several steps of precipitation and column chromatography, and are quite time consuming [2,7,8]. As more PAT-containing transgenic crops are being developed, a simple method allowing rapid isolation of protein from plant materials achieving a high degree of purity would be beneficial. Taking into account the binding capacity of PAT to acetyl-CoA [2,9,10], we wanted to test whether affinity chromatography using acetyl-CoA or an analog would work for purification of PAT.

A search for acetyl-CoA-based affinity purification methods found that reactive dyes have been successfully used for cofactor-dependent protein purification [11–13]. Most of the reactive dyes used in dye-affinity systems such as Cibacron blue 3GA and Reactive brown 10 consist of a sulfonated chromophore linked to a chlorotriazine group by an aminoether bridge. Molecular modeling has shown a rough resemblance between Cibacron blue F3G-A and NAD, but the most important similarities are with the planar ring structure and the negative charge groups [13]. Reactive brown 10, similar to acetyl Co-A, has a carboxyl group which is absent in other commonly used reactive dye resins. Experimentally, it has been demonstrated that reactive dyes can mimic the natural nucleotide cofactors to interact with cofactor binding sites of protein [13–15]. Thus, reactive dyes can be used as affinity ligands for cofactor-dependent protein purification [13]. In an earlier study, a reactive dye-based affinity chromatography procedure was developed to purify acetyl-CoA-dependent pyruvate carboxylase from the photosynthetic bacterium *Rhodobacter capsulatus* [12]. Cibacron blue 3GA has been shown to bind to dehydrogenases [16], kinases [17], restriction endonucleases [18] while Reactive red 120 has been used for the isolation of NADP dependent dehydrogenases [19]. It was also reported that Reactive brown 10, along with other reactive dyes, can be used for purification of

* Corresponding author. Fax: +1 314-694-9113.
E-mail address: cunxi.wang@monsanto.com (C. Wang).

[1] *Abbreviations used:* PAT, phosphinothricin *N*-acetyltransferase; L-PPT, phosphinothricin.

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glycosyltransferases [14]. In addition, reactive dyes are stable, easy to be immobilized, inexpensive and commercially available. In this study, we screened immobilized CoA, acetyl-CoA, and five commonly used reactive dye resins including Cibacron blue 3GA, Reactive red 120, Reactive green 19, Reactive blue 4, and Reactive brown 10 to identify those which were able to effectively purify PAT from both *Escherichia coli* and plant extracts. We found that Reactive brown 10 can be used as a pseudo-substrate to purify the PAT protein from multiple sources.

Materials and methods

Cloning, expression, and extraction from E. coli

The *bar* coding sequence (GenBank Accession No. X05822) was amplified by PCR and ligated into pET23b vector (Novagen, Madison, WI).

The *E. coli* BL21 (DE3) strain harboring pET23 bar was grown in 1 L auto-induction medium (EMD, Gibbstown, NJ) supplemented with 50 μg/mL carbenicillin. Cells were grown at 37 °C with rotary shaking (250 rpm), until the culture OD_{600} reached ~0.7, at which point the temperature was reduced to 15 °C and cultures grown for an additional 48 h with continuous shaking. Cells were harvested by centrifugation (5000*g* for 20 min). The cell pellet was resuspended in 20 mM Tris, pH 8.0 at approximately a 1:20 sample weight to buffer volume ratio and disrupted by a cell disrupter at 25 Kpsi. The cell lysate was clarified by centrifugation at 25,000*g* for 15 min at 4 °C. The supernatant was collected for protein purification.

Resin screening

Five reactive dye agaroses, including Cibacron blue 3GA, Reactive red 120, Reactive green 19, Reactive blue 4, and Reactive brown 10-agaroses (Sigma–Aldrich, St.Louis, MO), were tested for use in purification of the PAT protein from *E. coli*. CoA agarose (Sigma–Aldrich, St. Luois, MO) was also tested, as was acetyl-CoA linked to AminoLink agarose resin (Thermo Scientific, Rockford, IL). These agaroses were washed with 20 mM Tris, pH 8.0. The washed agarose was added into the *E. coli* extract at approximately a 1:5 agarose to extract volume ratio. The agarose mixture was incubated at room temperature for 30 min with a gentle rotation. After incubation, the agarose was collected by centrifugation at 1000*g* for 1 min. The collected agarose was washed 3 times with 20 mM Tris, pH 8.0, 3 times with 1.5 M NaCl, 20 mM Tris, pH 8.0, and 1 time with 20 mM Tris, pH 8.0, respectively. Unless otherwise specified, the bound protein was eluted with two agarose column volumes of 20 mM Tris, pH 8.0, containing a combination of 1 mg/mL glufosinate and 1 mg/mL acetyl-CoA.

Protein purification from transgenic cottonseed

Approximately 50 g of pre-chilled transgenic cottonseed expressing the *bar* coding sequence were ground using a laboratory mill. The ground powder was defatted 3 times with 200 mL each time of hexane, air-dried, and stored in a −80 °C freezer prior to extraction of the PAT protein. A portion of the defatted seed powder (10 g) was mixed with an extraction buffer (20 mM Tris, pH 8.0) at approximately a 1:10 powder weight to extraction buffer volume ratio and then incubated for 2 h with a gentle rotation at 2–8 °C. The slurry was centrifuged at 20,000*g* for 10 min at 4 °C. The resultant supernatant was subjected to $CaCl_2$ fractionation by addition of 0.85 mL of 1 M $CaCl_2$ to precipitate a significant proportion of the cottonseed storage proteins. The mixture was incubated on wet ice for 0.5 h and then centrifuged at 20,000*g* for 20 min. The supernatant was collected and mixed with 0.7 mL of Reactive brown 10-agarose that had been pre-equilibrated with 20 mM Tris, pH 8.0. After 1 h incubation at 4 °C with a gentle rotation, the mixture was centrifuged for 1 min at 1000 rpm and the agarose was collected. The agarose was washed with a buffer containing 20 mM Tris, pH 8.0, 1.5 M NaCl. The PAT protein was eluted with a buffer containing 0.5 mM acetyl-CoA, 20 mM Tris, pH 8.0. The protein eluted from the Reactive brown 10 was concentrated using a Nanosep Centrifugal Device (10 kDa cutoff, Pall Corporation, Ann Arbor, MI).

SDS PAGE electrophoresis

The sample was mixed with 5× loading buffer [312 mM Tris–HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 50% (v/v) glycerol, pH 6.8)], heated at 95 °C for 3 min, and applied on a pre-cast Tris glycine 4–20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Protein bands were visualized by InstantBlue stain (Expedeon Protein Solution, Babraham Hall, UK).

N-terminal sequencing

After electrophoresis, the PAT protein was electrotransferred to a PVDF membrane (pore size: 0.45 μm, Invitrogen, Carlsbad, CA) in a buffer containing 10 mM CAPS, pH 11 and 10% methanol at a constant voltage of 30 V for 1 h. Protein bands were visualized with Ponceau S stain (Sigma–Aldrich, St. Louis, MO) and subjected to N-terminal sequencing [20] via Edman degradation.

PAT activity assay

The PAT enzymatic activity assay was conducted as described previously [2].

Results

Reactive brown 10 enabled one-step purification of PAT from E. coli

The PAT protein was highly expressed in *E. coli* BL21 (DE3) at 15 °C in auto-induction medium. After cells were disrupted, the PAT protein was mainly in the supernatant (Fig. 1A, Lane 2). This *E. coli* lysate supernatant was incubated with CoA agarose and acetyl-CoA agarose. No binding of PAT was found to either CoA or acetyl-CoA agarose. Subsequently, five reactive dye agaroses were investigated for purification of the *E. coli*-expressed PAT. As shown in lane 4 of Fig. 1A, the flow-through fraction from Reactive brown 10 showed reduced stained band intensity at the expected position of PAT protein (~25 kDa) compared to other agarose flow-through fractions, indicating that Reactive brown 10 had the strongest ability to bind PAT among tested reactive dyes. After agaroses were washed extensively using a buffer containing 1.5 M NaCl, proteins were eluted with a buffer containing both glufosinate and acetyl-CoA (i.e. the substrates for PAT). As shown in lane 9 of Fig. 1A, a single high intensity band at ~25 kDa was detected in the fraction eluted from Reactive brown 10. In contrast to Reactive brown 10, little PAT protein was eluted from Cibacron blue 3GA and Reactive red 120 (Lanes 8 and 10, Fig. 1A), and no protein was detected in elutions from Reactive green 19 and Reactive blue 4 agaroses (Lanes 11 and 12, Fig. 1A). To confirm the protein identity, N-terminal sequencing of ~25 kDa protein eluted from Reactive brown 10 was performed. Sequence obtained for the protein was identical to the N-terminus of the PAT protein deduced from the coding sequence (Fig. 1B), confirming that the ~25 kDa protein was PAT protein. These results showed that