Specific Incorporation of Unlabeled Aromatic Amino Acids into ^{15}N-Labeled Sac7d Through use of Glyphosate

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Abstract

Selective labeling of amino acids can aid solution NMR studies of proteins by simplifying the assignments of resonances to residues. This is most valuable when assigning resonances for large proteins and in protein-substrate complexes. By growing bacteria in ^{15}N labeled media and in the presence of glyphosate, a shikimate inhibitor, we were able to eliminate production of the 7.6-kDa protein sac7d. When fed unlabeled Phenylalanine(F), Tyrosine(Y), and Tryptophan(W), the expression host produced normal levels of sac7d with <30% labeling of the aromatic residues.

Introduction

The protocol in this research used the DNA-binding protein sac7d from the hyperthermophilic archaeon *Sulfolobus acidocaldarius*; it was expressed in the *E. coli* strain Rosetta(DE3). The 66 residues of sac7d contain 5 aromatic amino acids. The solution NMR structure has been well resolved and its thermophilic nature simplifies the process of purification. Glyphosate is a widely used shikimate inhibitor which will prevent the *in vivo* production of aromatic amino acids and any proteins with aromatic residues. Feeding F, Y, and W, to inhibited cells allows protein production to occur.

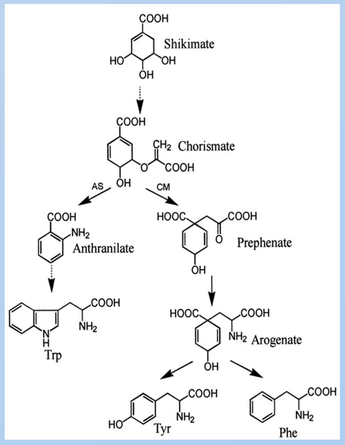

Fig. 1 - Demonstrates the shikimate pathway for biosynthesis of the aromatic amino acids: Phenylalanine(F), Tyrosine(Y), and Tryptophan(W).

Fig. 2 – Comparison of control (left) vs inhibited (right) 125-mL cultures in minimal media. At OD_{600} 0.4 glyphosate (2.5 mL) was added to sample. Picture taken after 4 hours.

Method

The *Escherichia coli* strain, Rosetta(DE3), was used to express sac7d throughout the study. Starter cultures (<5 mL) were all grown in Luria Broth and large scale cultures (>200 mL) were grown in an M9 minimal media. Early studies were conducted to test for host response to glyphosate. Cultures were induced with 1 mM IPTG (1.93 mL/L) when cultures reached an optical density of 1.0, and protein production was halted after 1 hour. After 1 hour the amount of recoverable protein drastically declined due to the toxic effect of the protein binding to host DNA. When a sample was needed for NMR, the M9 salts were supplemented with $^{15}NH_4Cl$. Cells were lysed by sonication and then centrifuged at15000g. The supernatant was heated to 75˚C for 15 minutes to remove other proteins from solution. The clarified supernatant was dialyzed in a phosphate buffer and purified via FPLC using a Q-Sepharose column. Purified samples were dialyzed against DI water and concentrated for use in ^{1}H,^{15}N-HSQC.

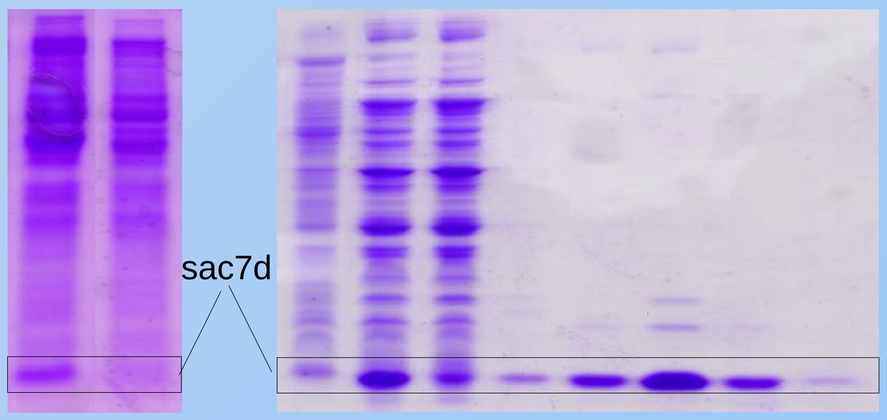

Fig. 3 (above left) Gel from control (left) and sample inhibited with 20 mL glyphosate/L of culture (right). Both samples were taken 1 hour after addition of IPTG.

Fig. 4 (above right) Gel from sample grown in ^{15}N minimal media. Lane 1 shows the total harvested sample. Lane 2 is the sample following heat cut and dialysis. Lane 3 was the wash from the FPLC, indicating that some of the protein did not bind to the column. The remaining lanes show the purified sample from the FPLC. The additional heavy proteins in lane 6 indicate a diminished level of purity in the sample but due to the small relative concentration caused minimal interference for the ^{1}H,^{15}N-HSQC spectrum and did not interfere with resonance assignment.

Results

Currently the best results were obtained from a 1-L culture grown in ^{15}N media, inhibited with 20-mL glyphosate at OD_{600} 0.4, and recovered with 500-mg unlabeled F, Y, and W. Subsequent purification and analysis of the protein revealed >70% reduction of labeled aromatic residues compared to the baseline determined by the control.

^{1}H,^{15}N-HSCQ

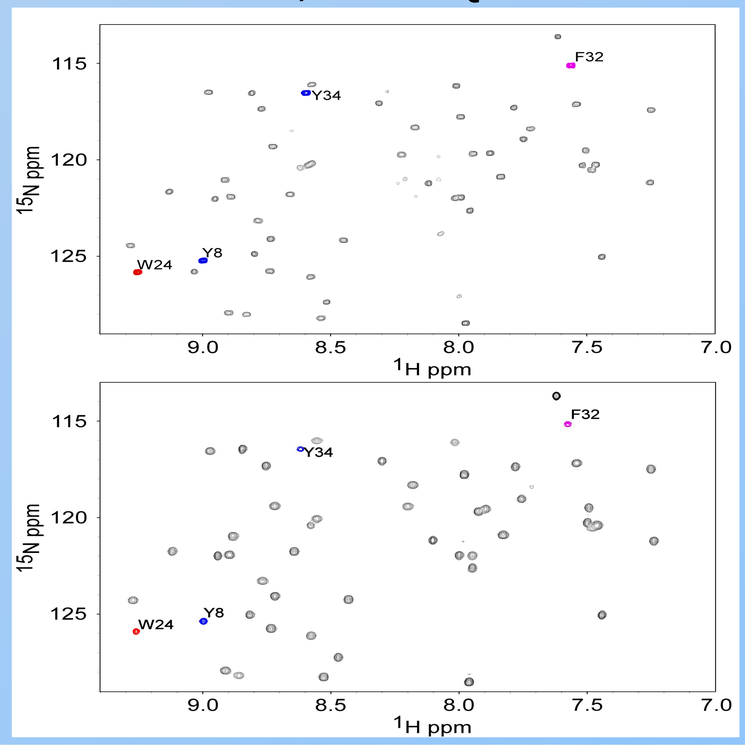

Fig. 5,6. 2D NMR spectra for sac7d. Top figure shows baseline spectrum from uninhibited culture. Bottom figure shows spectrum from inhibited and recovered culture. Aromatic amino acids colored for emphasis [Tryptophan (red), Tyrosine(blue), Phenylalanine (magenta)].

Further Research

- Refine procedure to >90% incorporation
- Reverse process with unlabeled media and ^{13}C- ^{15}N labeled F, Y, and W.
- Develop universal protocol for selective aromatic labeling in recombinant proteins

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