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Auto-induction medium containing glyphosate for high-level incorporation of unusual aromatic amino acids into proteins

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Abstract

We describe the use of an auto-induction medium containing N-(phosphono-methyl)glycine (glyphosate) as a means for high-level introduction of nonstandard aromatic amino acids into a protein. We illustrate this approach by preparing maltose binding protein (MBP) wherein all eight tryptophan residues have been replaced with 6-fluorotryptophan at an incorporation level of 99.3%. Such a high level of incorporation is important for spectroscopic investigations, in particular ^{19}F NMR, because each species' differing amino acid sequence potentially yields a different peak pattern that complicates spectral analysis.

Keywords

protein labeling; aromatic amino acid analogs; unnatural amino acids; fluorotryptophan

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Nonstandard amino acids commonly are introduced into proteins to test mechanistic hypotheses or to provide spectroscopic probes or heavy atoms used in phasing X-ray structures. The usual goals are to maximize the level of incorporation of the unusual amino acid and the overall yield of protein. Tryptophan analogs are of particular interest because of their spectral properties (1).

Incorporation of unusual amino acids into recombinant proteins produced from cells presents several obstacles. Replacement of natural residues in proteins by appropriate analogs usually is achieved by tightly controlled overproduction of the protein in an appropriate auxotrophic *Escherichia coli* strain growing in minimal medium with the desired unusual amino acids (1–3). Because the bacterial host is starved for the natural amino acid and supplemented with the analog—which may be toxic or may slow protein production—protein yields often are low. If a significant amount of the natural amino acid is required, its presence will lower the level of incorporation of the unusual amino acid (4,5). Furthermore, it may be necessary to identify an optimal host and then create an auxotrophic version of that strain. It may be possible to use cell-free protein production to overcome difficulties with toxicity (6), but this approach can be expensive if large amounts of protein are required. An elegant method for introducing unnatural amino acids is to make use of an engineered aminoacyl-tRNA synthetase and cognate orthogonal tRNA that recognizes the amber codon (7,8). Another method utilizes induction of an mRNA-specific endoRNase that shuts down

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Competing interests
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background protein synthesis while allowing production of the target protein and introduction of toxic amino acids (9).

A convenient approach to introducing unusual aromatic amino acids into proteins produced from *E. coli* cells involves the use of N-(phosphonomethyl) glycine (glyphosate), commonly used as an herbicide, to inhibit the biosynthesis of aromatic amino acids (10). Cell growth and protein production in the presence of glyphosate requires the provision of all three aromatic amino acids (phenylalanine, tyrosine, and tryptophan), and the strategy is to replace one or more of these with the cognate unusual amino acid(s) to be incorporated. This approach has been used to introduce fluorotryptophan into a protein (5), but the protein yield was modest, and the level of incorporation was limited to ~80%, because unlabeled amino acid had to be supplied to support cell growth.

Recent approaches to improving protein yields include cold-shock induction with pCold vectors (11), high cell density IPTG induction method (12), and the auto-induction medium introduced by Studier (13) as an alternative to batch induction by IPTG. The auto-induction medium contains components that are metabolized differentially and support cell growth to high density, at which point protein expression is induced automatically from a lac promoter. The auto-induction approach has been adopted and refined for the production of selenomethionine and stable isotope-labeled proteins for structural and functional studies (14–16).

We describe here the use of glyphosate in an auto-induction medium as an approach for increasing both the level of incorporation of a f luorine-labeled aromatic amino acid and the overall protein yield. The approach enabled the production of maltose binding protein (MBP), wherein all eight tryptophan residues were replaced by 6-fluorotryptophan at an incorporation level of 99.3% without the use of an auxotrophic strain of *E. coli*.

To evaluate and optimize the approach, we investigated the production of recombinant MBP in the presence of the unnatural aromatic amino acid 6-fluoro-D,L-tryptophan (6F-Trp). We compared the glyphosate-containing medium developed by Kim et al. (5), which utilizes IPTG induction, with a glyphosate-containing auto-induction medium [Studier MDA-5052 (13)]. We found that *E. coli* cells [BL21-Codon Plus(DE3)-RIL carrying pMAL-p2E] grew to high density ($OD_{600} \geq 8.0$ after 24 h) on the auto-induction medium and also in the presence of glyphosate ($OD_{600} \approx 7.5$ after 24 h), provided that aromatic amino acids were supplied (Figure 1). The rate of cell growth was nearly as high when 86% of the Trp was replaced by 6F-Trp, but with 100% 6F-Trp in the medium, the rate of cell growth fell significantly ($OD_{600} \approx 6.0$ after 24 h). As expected, the cells failed to grow in the presence of glyphosate without added tyrosine (Tyr) and Trp. The level of cell growth on the glyphosate-containing medium described by Kim et al. (5) was poor ($OD_{600} \approx 1$ after 20 h).

MBP was produced in high yield by the glyphosate-containing auto-induction medium but not when lactate was absent from the medium (noninducing medium) (Figure 2A). The amount of protein produced in the presence of glyphosate was only somewhat lower than in its absence (Figure 2B). 6F-Trp-MBP was purified by amylose resin affinity chromatography with elution by maltose and yielded a single major band on SDS-PAGE (Figure 2C). The average yield of purified 6F-Trp–labeled MBP, obtained after proteolysis of the fusion (MBP-lacZ-α) protein, was ~20–30 mg/L (50-mL working volume) for three expression trials (average $OD_{600} \approx 6.5$ at cell harvest; wet cell mass 8.7 g/L).

Analysis by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS), interpreted with the deconvolution algorithm MaxEnt (Figure 2D), gave 43,109.0 Da (versus calculated value of 43,100.4 Da) for the purified unlabeled MBP, and 43,252.0 Da (versus calculated value of 43,244.38 Da) for the purified labeled MBP (grown on 50 µg/L

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Trp and 316 mg/L 6F-Trp). The difference of 143.0 Da—in comparison to the predicted molecular weight difference of 143.98 Da for 100% labeling of the eight Trp residues—indicated that the incorporation rate was 99.3%.

The approach described herein should be applicable to the incorporation of a variety of aromatic amino acids. Its advantages are simplicity (no need for auxotrophs or induction), high protein yields, and high-level incorporation of the amino acid analog.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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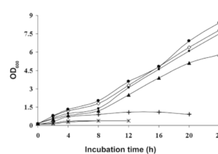

Figure 1. Time course of *E. coli* [BL21-Codon Plus(DE3)-RIL carrying pMAL-p2E] cells grown in 250-mL conical shaker flasks (225 rpm) at 30°C on 50 mL media of different composition
(●) Auto-induction medium with amino acid mixture I [all 20 amino acids except cysteine (Cys) and Trp] without added glyphosate. (◊) Auto-induction medium with glyphosate (1 g/L), amino acid mixture I, and Trp (200 µg/L). (■) Auto-induction medium with glyphosate (1 g/L), amino acid mixture I, 6F-Trp (316 mg/L), and Trp (50 µg/L). (▲) Auto-induction medium with glyphosate (1 g/L), amino acid mixture I, and 6F-Trp (316 mg/L). (×) Auto-induction medium with glyphosate (1 g/L), amino acid mixture I, and Tyr (200 µg/L). (⁎) Auto-induction medium with glyphosate (1 g/L), amino acid mixture II (all 20 amino acids except for Cys, Trp, and Tyr), and Trp (200 µg/L). (+) Kim et al. (5) medium (with IPTG induction) containing glyphosate and 6F-Trp.

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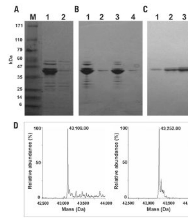

Figure 2. Expression and purification of MBP produced from *E. coli* [BL21-Codon Plus(DE3)-RIL] cells carrying pMAL-p2E

(A) SDS-PAGE analysis of extracts (lane 1) from cells grown in the auto-induction medium and (lane 2) from cells grown in the same medium without lactate [noninduction medium MDAG (13)]. Lane M contains a mixture of molecular weight markers with masses indicated. (B) SDS-PAGE analysis of cell extracts: (lane 1) soluble fraction; (lane 2) insoluble fraction from cells grown on the auto-induction medium with glyphosate (1 g/L), amino acid mixture I (all 20 amino acids except Cys and Trp), and Trp (200 μg/L); (lane 3) soluble fraction; (lane 4) insoluble fraction from cells grown on the auto-induction medium with glyphosate (1 g/L), amino acid mixture I, 6F-Trp (316 mg/L), and Trp (50 μg/L). (C) SDS-PAGE of 6F-Trp-labeled MBP purified by affinity chromatography. Lanes 1, 2, and 3 show three sequential fractions eluted with 10 mM maltose. (D) Deconvoluted ESI-mass spectra of purified MBP obtained from cultures grown in a chemically defined auto-induction medium for aromatic unusual amino acid incorporation: (left) unlabeled MBP and (right) 6F-Trp-MBP.