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Successful Expression of a Selenomethionyl Protein under Control of the Temperature-Sensitive λ Repressor Requires Higher than Normal Temperature

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Selenomethionyl proteins offer unique investigative opportunities in elucidation of protein three-dimensional structures either by multiwavelength anomalous diffraction and synchrotron radiation X-ray crystallographic analysis (4,5) or by nuclear magnetic resonance spectroscopy when the ^{77}Se isotope is used (10). Incorporation of selenomethionine into proteins is usually accomplished by growing a methionine auxotroph of *Escherichia coli*, which carries the expression plasmid, in a defined medium containing selenomethionine (3,12). However, yields of selenomethionyl proteins are often low, particularly when expression is under control of a temperature-sensitive repressor (1).

Thioesterase II, an enzyme that modifies the product specificity of the lipogenic pathway in mammary glands by releasing medium-chain fatty acids from the multifunctional fatty acid synthase (8,9), has been crystallized, and preliminary diffraction studies have been performed (2). In the pJLA502 vector used for expression of thioesterase II, transcription initiation is very effectively repressed at 28°–30°C by the temperature-sensitive λ repressor (13), and full induction of protein expression is normally achieved by shifting the incubation temperature to 42°C (11,13). In preliminary experiments, when the methionine auxotroph of *E. coli* DL41 cells ($F^-\lambda^-$ *metA*$^-$) (3) harboring the pJLA502/thioesterase II construct was grown in a defined LeMaster medium supplemented with 25 mg/L methionine (7) and 10 mL/L of Kao and Michayluk Vitamin Solution (100×; Sigma Chemical, St. Louis, MO, USA), 4 mL/L glycerol (replacing glucose), good expression of thioesterase II protein was observed at 42°C. On the other hand, when methionine was replaced with selenomethionine, only a trace amount of thioesterase II was produced at 42°C. When the temperature of the BioFlo IIc® fermentor (New Brunswick Scientific, Edison, NJ, USA) was incrementally raised from 38° to 44°C and samples were removed periodically for analysis, we found that significant amounts of the selenomethionyl form of the thioesterase were produced only after the fermentor reached 44°C (Figure 1). Shifting the temperature to 44°C did not increase expression of the normal methionyl thioesterase. The apparent requirement for a higher temperature to express the selenomethionyl form of the protein was confirmed by measuring directly the yield of enzyme produced by a 4-h induction at 42°C (0.5 ± 8.6 enzyme U/mL cytosol) and 44°C (65.3 ± 6.1 U/mL). More than 70% of

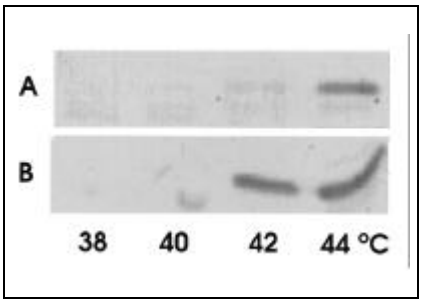

Figure 1. Western blot analysis of whole-cell lysates of DL41 cells carrying pJLA502 with thioesterase II cDNA. The starter culture was grown overnight at nonpermissive temperature in LeMaster medium (7) supplemented with 25 mg/L methionine and 10 mL/L of Kao and Michayluk Vitamin Solution (100×), 4 mL/L glycerol (replacing glucose). A portion of the culture was transferred to the fermentor, which had been equilibrated to 30°C and contained 100 vol of LeMaster medium supplemented with vitamins and 25 mg/L selenomethionine (A) or methionine (B). The temperature of the fermentor was maintained automatically at the preset value, ±0.1°C, and confirmed by manual reading of a thermometer positioned in contact with the medium. The temperature was initially maintained at 30°C for 2 h, reset to 36°C and then increased stepwise by 2°C every hour. When the temperature reached 44°C, incubation was continued for 1 h before sampling. At each temperature, an equal cell mass based on OD_{600} was centrifuged at 12 000× *g* for 5 min, cells were lysed by boiling in 6 M urea and 1% SDS, and the extracted proteins were separated on an SDS 12% polyacrylamide discontinuous gel (6) and analyzed by Western blotting using rabbit anti-rat thioesterase II antibodies.

the selenomethionyl thioesterase II protein produced at 44°C was soluble (data not shown). Yields of both the selenomethionyl and normal recombinant proteins were improved significantly using glycerol rather than glucose as the carbon source. These data indicated that derepression of transcription initiation requires higher temperature when methionine auxotroph *E. coli* cells are grown in a medium containing selenomethionine.

To achieve a high content of selenomethionine in the recombinant protein, the fermentor containing 2.1 L of modified LeMaster medium equilibrated to 30°C was seeded with 90 mL of an overnight starter culture that had been grown in the LeMaster medium containing 80% selenomethionine and 20% methionine. When the optical density $(OD)_{600}$ of the fermentor medium reached 0.3, the temperature was reset to 44.5°C. In the fifth hour of induction, the medium was augmented with additional glycerol (0.5 mL/L) and ammonium phosphate (2 g/L) to ensure continued cell growth. Cells were harvested after 7 h of induction, before reaching stationary phase, when typically the OD_{600} reached about 4. Under these conditions, we were able to produce 1.3 mg of soluble selenomethionyl thioesterase II per liter of the cell culture (4.5 g of cells). About 40%–50% of the yield of soluble normal enzyme was achieved in the presence of methionine. A similar comparison published for the expression of phosphomannose isomerase at 42°C shows less than a 1:10 yield ratio for selenomethionyl and methionyl protein (1).

For characterization of the selenomethionyl thioesterase II, cells were lysed in 0.1 M Tris-HCl, pH 8.0, 10 mM EDTA, 5 mM dithiothreitol and 5 mM 2-mercaptoethanol (1:3, wt/vol) by two passages through a French press, and the selenomethionyl thioesterase II was partially purified (11,15). All buffers contained 1.5 mM dithiothreitol and 1 mM 2-mercaptoethanol. These

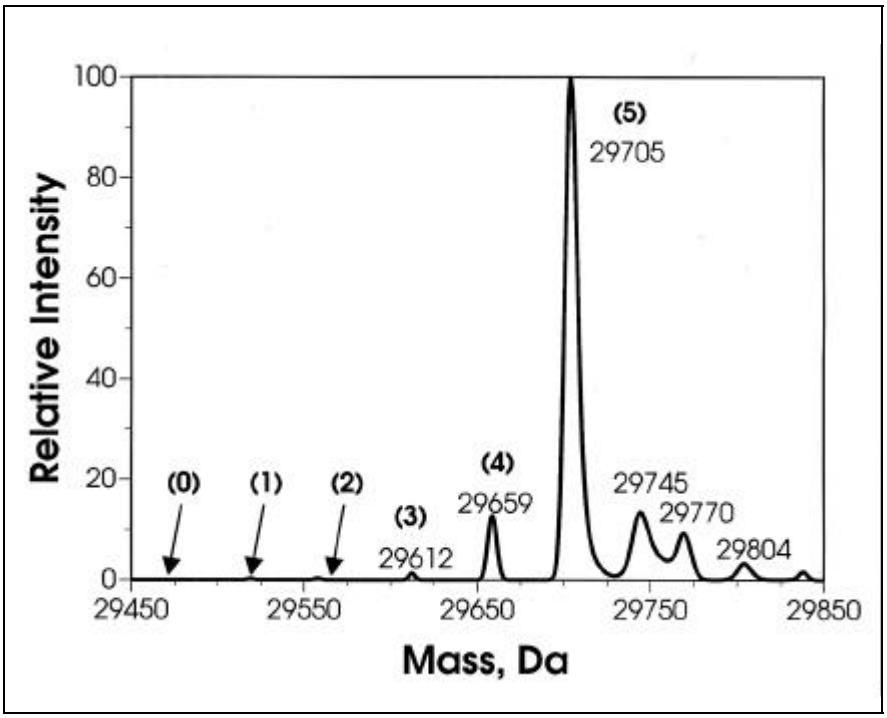

Figure 2. Reversed-phase HPLC/ESI-MS analysis of selenomethionine thioesterase II. ESI-MS was performed by reversed-phase HPLC (Narrow Bore HPLC System; Michrom BioResources, Auburn, CA, USA) with purified enzyme essentially as described previously (14). Multiple 10-s scans acquired within a 935–1140 m/z range were combined to produce the final spectrum. Number of selenomethionine residues incorporated into thioesterase II is shown in the parentheses. The molecular mass expected for the normal thioesterase II is 29 471 Da, and those for the enzyme containing five, four and three selenomethionine residues are 29 706, 29 659 and 29 612 Da, respectively. The identity of the species having molecular masses >29 705 Da, which likely represent contaminants, was not determined.

thiol compounds were included in both the lysis medium and the buffers used in protein purification primarily to deter oxidation of selenomethionine residues, but also to protect cysteine thiol residues in the enzyme. The content of selenomethionyl residues in the thioesterase II was analyzed by electrospray ionization mass spectrometry (ESI-MS; VG BioQ Mass Spectrometer; Micromass, Altrincham, England, UK) (Figure 2). In 91.5% of the purified enzyme (86.5% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]), all five methionine residues were replaced with selenomethionine, 7.9% contained four selenomethionine residues and 0.6% contained three. Thus, the contribution of normal methionine carried over from the starter culture amounted to only 1.8% of the methionine residues overall. This level of replacement by selenomethionine is considered quite acceptable for crystallographic analysis. Specific enzyme activities of the selenomethionine thioesterase II with model and natural substrates (9) were indistinguishable from that of the normal enzyme.

In conclusion, when recombinant thioesterase II was expressed in a selenomethionine-containing medium in a plasmid under control of the λ C_{I857} gene repressor, an increase in the induction temperature by two to three degrees improved by 100-fold the yield of soluble, fully functional enzyme with 98.2% selenomethionine replacement. These data suggest that thermal stability of the λ repressor/operator complex may be increased when methionine residues are replaced by selenomethionine.

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