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SHORT COMMUNICATION

Investigation into the misincorporation of norleucine into a recombinant protein vaccine candidate

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Abstract A high level of norleucine misincorporation was detected in a recombinant methionine-rich protein vaccine candidate expressed in *E. coli* K12. An investigation was conducted to evaluate a simple remediation strategy to reduce norleucine misincorporation and to determine if the phenomenon was either (a) due to the depletion of methionine during fermentation, (b) a result of the cultivation environment, or (c) a strain-specific effect. While supplementation with exogenous methionine improved product quality, the undesirable biosynthesis of non-standard amino acids such as norleucine and norvaline persisted. In contrast, non-standard amino acid biosynthesis was quickly minimized upon selection of an appropriate fed-batch process control strategy, fermentation medium, and nutrient feed. By expressing the same protein in *E. coli* BL21(DE3), it was determined that the biosynthesis of norleucine and norvaline, and the misincorporation of norleucine into the protein were primarily attributed to the use of *E. coli* K12 as the host for protein expression.

Keywords Norleucine (Nle) · Norvaline (Nva) · Misincorporation · Chemically defined medium (CDM) · Semi-defined medium (SDM)

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Introduction

The biosynthesis and substitution of norleucine (Nle) for methionine (Met) in recombinant proteins is a well-known example of misincorporation of a non-standard amino acid caused by tRNA misaminoacylation. This phenomenon has been reported in various recombinant proteins expressed in *E. coli*, including interleukin 2, bovine somatotropin, human brain-derived neurotrophic factor, and human macrophage colony stimulating factor [3, 7, 8, 13, 16, 18, 20]. Other non-standard amino acid substitutions have been reported as a result of norvaline (Nva) biosynthesis and selection by leucine-tRNA, and β-methylnorleucine biosynthesis and selection by isoleucine-tRNA [2, 14, 15].

In this study, the presence of Nle was detected in a 41 kDa Met-rich recombinant protein vaccine candidate, where Met comprised 4.7 % of the total number of residues, when the *E. coli* K12 strain was used as the host for protein expression. Previous reports suggest that Nle can form as a byproduct of the leucine (Leu) amino acid biosynthetic pathway in *E. coli* and other Gram negative microorganisms [7, 10], and Nle substitutions are linked to low levels of Met in the culture medium, as well as a high Met content of the recombinant protein [7]. Numerous interventions have reportedly been successful in reducing and/or eliminating Nle substitution, including: supplementing the cultivation medium with free Met to increase the cellular Met to Nle ratio and promote accurate amino acid selection by aminoacyl-tRNA synthetase [7, 9]; reducing Nle biosynthesis by inactivating one or more genes in the *leu* operon [6]; co-expressing several enzymes such as glutamate dehydrogenase which are capable of degrading Nle or other non-standard amino acids [1]; overproducing Met by mutating genes involved in Met biosynthesis and regulation (*metA*, *metK*, and *metJ*) [21]; and supplementation

of molybdenum, nickel and selenium under fermentation conditions of limited oxygen and excess glucose [5]. While re-engineering the host strain for protein expression may not be feasible for a specific vaccine candidate already in development, exogenous amino acid or trace element supplementation is a viable option to improve product quality, although this can increase the complexity and cost at production scales. In our study, Met supplementation to the culture medium reduced non-standard amino acid substitution in the recombinant protein target, but did not reduce the biosynthesis of the non-standard amino acids Nle and Nva. Conversely, modification of the cultivation environment through alternative fed-batch process control or alternative fermentation medium and nutrient feed resulted in reduced biosynthesis of both non-standard amino acids. In addition, the biosynthesis of Nle and Nva decreased when the vaccine candidate was expressed in *E. coli* BL21(DE3).

Materials and methods

Expression strains

The two recombinant *E. coli* strains K12 and BL21(DE3) used in this study harbored isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible expression plasmids for the production of a 41 kDa recombinant protein vaccine candidate.

Fermentation conditions and protein purification

Recombinant *E. coli* was grown in a 2 L B.Braun fermenter containing either: (1) semi-defined medium (SDM) and feed (both containing yeast extract); or (2) Korz-based chemically defined medium [11] (CDM) and feed. Air was supplied at 3.0 L/min and the temperature was maintained at 30 °C. The fermentation was run in batch mode until glucose was depleted, after which fed-batch nutrient feeding was initiated using either a pH–stat or DO–stat fed-batch control scheme. Under pH–stat fed-batch control, the dissolved oxygen level (dO_2) was maintained at 30 % of air saturation by cascading the impeller rotational speed between 700 and 1000 rpm. When DO–stat fed-batch control was used, the impeller rotational speed was constant at 880 rpm and the dO_2 was maintained at 30 % using a feedback control mechanism to adjust the nutrient feed rate. Culture optical densities at 600 nm (OD_{600}) were measured offline using a spectrophotometer. When the culture reached an OD_{600} of approximately 40, recombinant protein expression was induced by the addition of IPTG for 6 h, after which the cells were harvested and the recombinant protein vaccine candidate inclusion bodies (IBs) were isolated by cell disruption and clarification. IBs were solubilized with 8 M urea and purified from either sodium dodecyl sulfate polyacrylamide gels by band excision and passive elution or by membrane chromatography.

Protein quantitation

Cell pellets were disrupted using an ultrasonic processor to release the target protein contained in IBs. The IBs were recovered by centrifugation, and then solubilized using a strong chaotrope. A Caliper LabChip® GXII (Perkin-Elmer) was used to estimate recombinant protein expression levels.

Amino acid analysis

One milliliter samples of either fermentation supernatant or purified vaccine candidate were sent to the SPARC BioCentre at The Hospital for Sick Children (Toronto, Canada) for amino acid analysis. Samples were dried and hydrolyzed with HCl/phenol for 24 h at 110 °C prior to derivatization by phenylisothiocyanate and HPLC analysis using a modified Pico-Tag gradient at 48 °C. The derivatized amino acids were detected at 254 nm. The data were collected, stored, and processed using Waters Empower™ 3 Chromatography software.

Mass spectrometry

Prior to LC–MS/MS analysis, protein samples were reduced with dithiothreitol, alkylated with iodoacetamide, digested with trypsin overnight at 37 °C, and quenched with 10 % trifluoroacetic acid. Digested samples were diluted 1:20 in 0.1 % formic acid and analyzed on a LC-ESI-Q-TOF-MS (LC–MS/MS) system (Waters Corporation). Data were analyzed with Mascot v2.4 (http://www.matrixscience.com) to determine peptide compositions.

Results and discussion

Supplementation of Met reduces Nle substitution but not its biosynthesis in a Met-rich vaccine candidate

SDM and nutrient feed containing yeast extract was used to cultivate and induce the expression of a recombinant vaccine candidate in *E. coli* K12 under a pH–stat fed-batch control strategy. Mass spectrometric analysis by LC–MS/MS and amino acid analysis by HPLC revealed high levels of Nle incorporation at random Met sites in the purified vaccine candidate. The relative amount of Met to its structural analog, Nle, varied from 23 to 49 % between multiple fermentations (Table 1, Runs 1–4). Nva misincorporation was not detected in the purified protein (data not included). Amino acid analysis of fermentation supernatant showed depletion of Met and Leu prior to and after induction of the target protein, with reciprocal accumulations of Nva and

Table 1 Summary of the Nle substitution results obtained from analysis of the purified vaccine candidate by HPLC (amino acid analysis) and mass spectrometry

Run	Medium, control scheme, *E. coli* strain	Met supplementation	Mass spectrometry (No. of modification sites detected)	Amino acid analysis (Met + Nle = 100 %)	
				Met	Nle
1–4	SDM, pH–stat, K12	No	12–15	50.8–77.2	22.8–49.2
5–7	SDM, pH–stat, K12	Yes	0*-1	94.4–98.5	1.5–5.6
8–10	CDM, DO–stat, K12	No	NA	97.8–98.5	1.5–2.2
11	SDM, pH–stat, BL21(DE3)	No	NA	99.2	0.8

Fermentation conditions tested were: pH–stat fed-batch control using SDM with and without Met supplementation, and DO–stat fed-batch control using CDM without Met supplementation

(*NA* not analyzed)

* Below threshold of detection

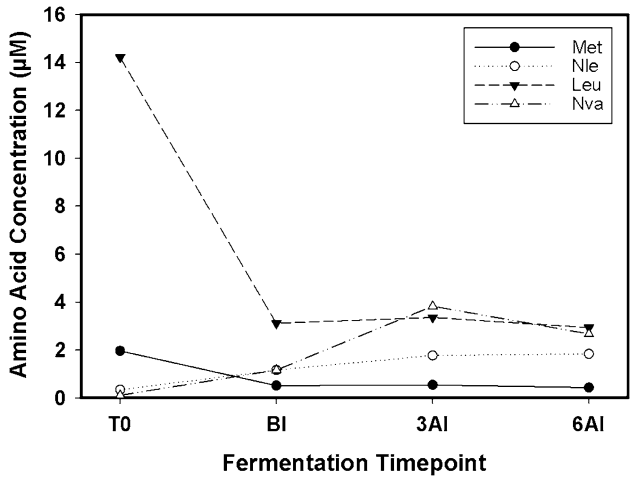

Fig. 1 Amino acid analysis of samples of clarified *E. coli* K12 fermentation medium revealed the depletion of Met and Leu over time and the corresponding accumulation of Nle and Nva. *T0* start of fermentation, *BI* before induction, *3AI* 3 h after induction, *6AI* 6 h after induction

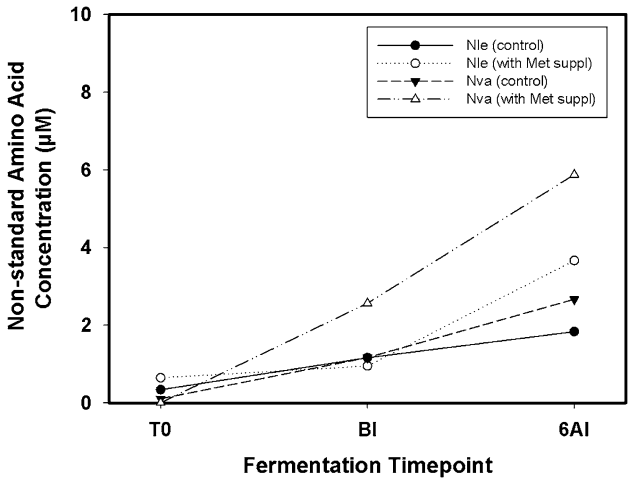

Fig. 2 Accumulation of Nle and Nva in the fermentation medium during pH–stat fed-batch cultivation of *E. coli* K12 in SDM with and without Met supplementation (with Met suppl). *T0* start of fermentation, *BI* before induction, *6AI* 6 h after induction

Nle from the *E. coli* K12 strain (Fig. 1). At 6 h post-induction, the molar level of Nle in the fermentation medium was fourfold higher than Met, while the molar level of Nva was about the same as Leu. Supplementation of free Met to both the fermentation medium and nutrient feed stream successfully reduced Nle misincorporation into the vaccine candidate (Table 1, Runs 5–7); however, the undesirable biosynthesis of Nle and Nva persisted and remained a production risk during the fermentation process (Fig. 2). Supplementation with free Leu did not have an impact on the percent Nle incorporation in the vaccine candidate (data not shown).

The fed-batch process control strategy and nutrient composition can minimize Nle and Nva production

The combined effect of the fed-batch process control strategy and the cultivation medium on Nle and Nva accumulation in fermentation medium was evaluated with the *E. coli* K12 strain expressing the vaccine candidate in the absence of Met supplementation. Two different fermentation medium and nutrient feed combinations were evaluated: (1) a semi-defined base medium (SDM) and nutrient feed (both containing yeast extract), and (2) a chemically defined, Korz-based medium [11] (CDM) and nutrient feed. After the initial 5 h fed-batch phase of fermentation, the nutrient was fed to the bioreactor under a pH or dissolved oxygen feedback control strategy (i.e., pH–stat or DO–stat, respectively).

Figure 3 shows that a 0.7–2.7 µM accumulation of Nle and Nva in the fermenter medium was observed with pH–stat fed-batch control regardless of the medium and nutrient feed strategy. Conversely, when DO–stat fed-batch control was used, both medium and nutrient feed strategies showed a decrease in Nle and Nva accumulation in the supernatant compared to pH–stat, with CDM and nutrient

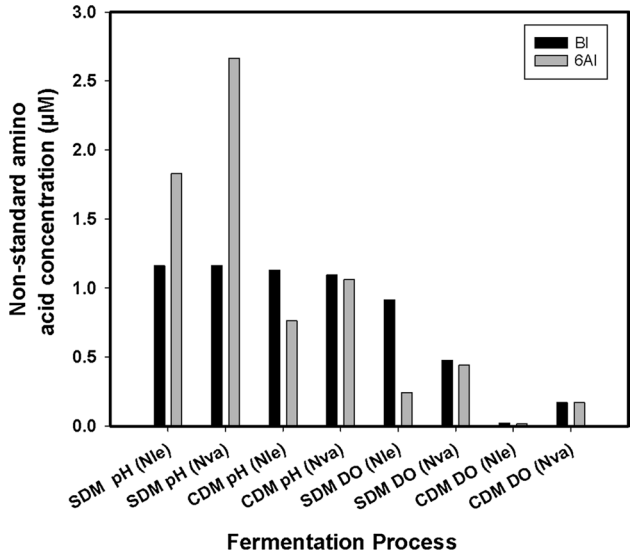

Fig. 3 Levels of Nle and Nva in the medium obtained from fermentation runs of *E. coli* K12 before induction (BI) and 6 h after induction (6AI) using a pH–stat or a DO–stat fed-batch control strategy and SDM or CDM. Met was not supplemented to any cultures

Table 2 Ratio of Met to Nle and Leu to Nva in the fermentation medium before induction (BI) and 6 h after induction (6AI) from *E. coli* K12 fermentation runs using a pH–stat or DO–stat fed-batch control strategy and SDM or CDM

Control scheme, medium and feed	Fermentation time point	Met:Nle	Leu:Nva
pH–stat, SDM	BI	1.0:2.0	3.0:1.0
pH–stat, SDM	6AI	1.0:3.7	1.2:1.0
DO–stat, SDM	BI	1.0:1.5	16:1.0
DO–stat, SDM	6AI	2.2:1.0	17:1.0
DO–stat, CDM	BI	5.0:1.0	15:1.0
DO–stat, CDM	6AI	10:1.0	16:1.0

Met was not supplemented in these experiments

feed (0–0.2 μM) showing a lower accumulation compared to SDM and nutrient feed (0.2–0.9 μM) (Fig. 3). Indeed, HPLC analysis of the vaccine candidate purified from fermentations using CDM combined with a DO–stat fed-batch control showed low levels of Nle incorporation comparable to those observed in fermentations using pH–stat fed-batch control and SDM supplemented with Met (Table 1, runs 8–10). This is consistent with the fermentation supernatant analysis that showed a higher ratio of Met:Nle and Leu:Nva in *E. coli* K12 fermentations using CDM and nutrient feed with DO–stat fed-batch control compared to using SDM and nutrient feed with pH–stat fed-batch control (Table 2).

The biosynthesis of Nle and Nva is reportedly related to excess levels of glucose and high levels of pyruvate within the cells under oxygen-limited conditions; this results in

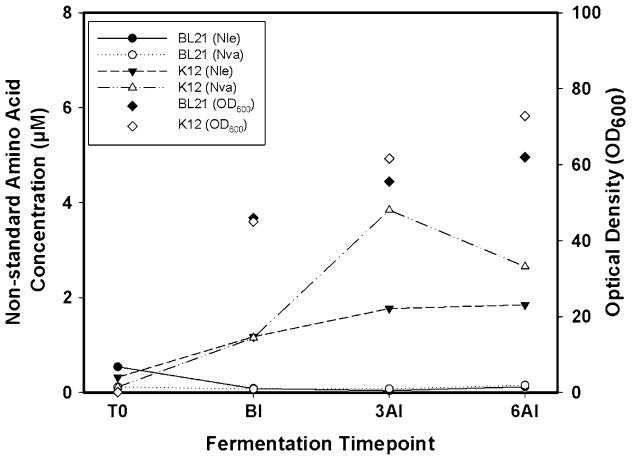

Fig. 4 Biomass and levels of Nle and Nva in the fermentation medium produced by *E. coli* K12 and *E. coli* BL21(DE3) strains throughout the course of fermentation using SDM and pH–stat fed-batch control strategy without Met supplementation

overflow metabolism and funneling of pyruvate to the synthesis of branched amino acids [17]. In agreement with this report, glucose excess (due to the pulsed addition of nutrient feed) and oxygen limitation are common throughout pH–stat fed-batch control fermentation, and thus a higher accumulation of Nle and Nva is seen in the fermentation medium. Conversely, in DO–stat fed-batch control, glucose is limited by the controlled addition of glucose in the nutrient feed and dO_2 is maintained at 30 % throughout the fermentation. This situation appears to correlate with a lower accumulation of Nle and Nva in the supernatant and is further improved when CDM and nutrient feed are used.

The effect of Nle incorporation was primarily attributed to the expression strain

Reports on the biosynthesis of Nva and Nle in *E. coli* appear to be limited to *E. coli* K12 and its sub-strains such as W3110G [7, 12, 17, 20]. To investigate whether the biosynthesis of Nle and Nva was a strain-specific phenomenon, a side-by-side comparison of the recombinant *E. coli* K12 and *E. coli* BL21(DE3) strains expressing the vaccine candidate was conducted in a pH–stat fed-batch fermentation using SDM and nutrient feeds without Met supplementation. Compared to the *E. coli* K12 strain, a low accumulation of Nle and Nva was measured in the fermentation supernatants from the *E. coli* BL21(DE3) strain (Fig. 4), which correlated with lower Nle and no Nva incorporation in the purified vaccine candidate protein (Table 1, Run 11 and data not shown). Together, this provides an additional approach to eliminating the production and substitution of Nle and Nva in recombinant proteins of interest, and in this particular case, without a dramatic loss in expression level.

Conclusions

This study found that the depletion of Met in the fermentation medium of high-density cultures of *E. coli* K12 resulted in a high level of Nle misincorporation in a recombinant protein vaccine candidate. While supplementation of the fermentation medium with Met reduced Nle misincorporation, the biosynthesis of Nle and Nva persisted. Further investigation showed that using CDM and nutrient feed in combination with DO–stat fed-batch control could reduce biosynthesis of these two non-standard amino acids without the need for exogenous amino acid supplementation. Lastly, we found that the level of Nle and Nva biosynthesis and Nle substitution at the protein level could be dependent on the host expression system, as non-standard amino acid biosynthesis and incorporation were substantially reduced when *E. coli* BL21(DE3) was used instead of *E. coli* K12. Together, the conclusions of this investigation may be of practical relevance for the selection of host *E. coli* strains and the fermentation process control strategy for recombinant protein production.

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