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Optimized Azidohomoalanine labeling protocol enables sensitive detection of *de Novo* protein synthesis in *C. elegans* under heat shock

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Abstract

Living organisms continually adjust protein expression in response to environmental stimuli. To systematically characterize this regulation, we analyzed the *C. elegans* nascent proteome using L-azidohomoalanine (AHA), a noncanonical methionine analog. Applying AHA to profile de novo protein synthesis during heat shock revealed stimulus-dependent changes in nascent protein production. Although AHA has been used previously to label newly synthesized proteins (NSPs) in *C. elegans* and enable bioorthogonal tagging via click chemistry, the labeling process itself has not been thoroughly optimized, limiting comprehensive proteomic analysis. Here, we present a modified exploratory study protocol—distinct from that reported in the literature—that improves AHA incorporation by optimizing the metabolic labeling of the *E. coli* food source. Specifically, inducing *E. coli* into AHA-containing medium at an OD of 0.2 (logarithmic phase), rather than the conventional OD 1.0 (stationary phase), significantly enhanced AHA labeling efficiency in *C. elegans*. This optimization increased AHA incorporation into the nascent proteome and yielded stronger post-click-chemistry affinity tag signals, enabling a more complete characterization of protein-regulation dynamics during heat shock. These exploratory findings define improved environmental conditions for efficient bioorthogonal labeling and provide an optimized workflow for future studies. Subsequent exploratory experiments demonstrated reliable *in vivo* identification of NSPs in *C. elegans,* supporting sensitive and direct analysis of protein synthesis under varying environmental stimuli.

Keywords

Bio-orthogonal, Optimization, Newly synthesized proteins, Environmental stimuli, Heat shock, Click chemistry, Labeling efficiency, Enrichment, Proteomics, Bioorthogonal noncanonical amino acid tagging

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1. Introduction

Cells constantly experience changes to their internal and external environment, and in response to these stimuli, proteins are dynamically regulated. New proteins are synthesized while others degrade to maintain cell homeostasis. Nascent proteomics provides a new method of analysis to identify newly synthesized proteins (NSPs) within the pre-existing protein population instead of analyzing the whole proteome (1). As such, these methods are more efficient and sensitive, allowing for selective identification of NSPs in response to varying degrees of stimuli (2).

NSPs are selectively tagged with amino acid isotopologues, noncanonical amino acids (ncAAs), or puromycin analogs that can be metabolically incorporated during protein synthesis. The process of bioorthogonal labeling with ncAAs allows NSPs to be isolated from the pre-existing protein population with high sensitivity. Subsequent conjugation with fluorescent dyes or affinity tags via bioorthogonal reactions such as click chemistry allows these dynamic proteomic changes in response to biological stimuli to be visualized and regulated (3). These methods have advanced the field of proteomics, allowing researchers to analyze protein abundance alterations due to different internal and external environmental changes.

Bioorthogonal noncanonical amino acid tagging (BONCAT) with L-azidohomoalanine (AHA) is not toxic to cells and provides stable labeling, since AHA is a noncanonical amino acid analog of methionine that can be introduced to cultured cells and incorporated into proteins during protein synthesis (4). However, optimizing the efficiency of such labeling has yet to be examined. In this study, we explore this research gap, revealing modified experimental conditions that significantly improve bioorthogonal labeling efficiency and analysis.

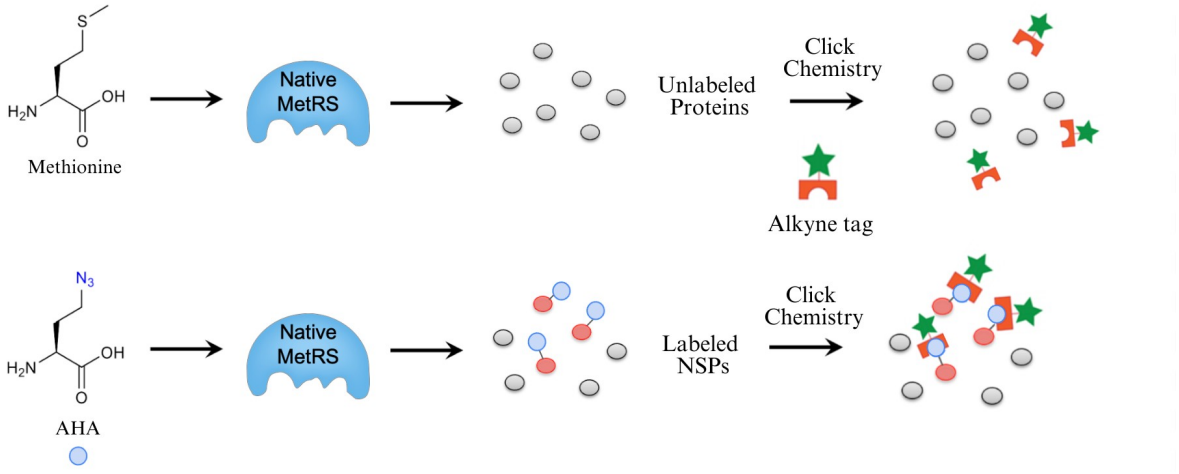

Figure 1. Bioorthogonal labeling workflow of L-azidohomoalanine (AHA). AHA is attached *in vivo* to methionyl-tRNA synthetase at the methionine site, allowing for AHA incorporation into *C. elegans* NSPs. Subsequent conjugation of AHA with various tags via click chemistry enables further analysis of the nascent proteome.

AHA, containing an azide group, is one of the most frequently used unnatural amino acids in nascent proteomics, attributed to the fact that AHA can be attached to methionyl-tRNA by native methionyl-tRNA synthetase and subsequently incorporated into NSPs at the methionine site (1). Methionine, unlike AHA, does not contain a bioorthogonal group and is therefore unable to perform similarly in a Cu-catalyzed alkyne-azide cycloaddition (CuAAC) reaction with affinity tags containing an alkyne group (Figure 1). Thus, AHA substitutes methionine to enable the labeling of the nascent protein.

Caenorhabditis elegans (C. elegans), a model organism highly conducive to research for its many advantageous features, is widely used to study proteomics. It feeds on bacteria, making it easy to culture in the lab; it reproduces rapidly; it is small in size; it is transparent, enabling the use of fluorescent markers *in vivo*; it is a complex multicellular organism with many different organs and tissues; and in *C. elegans*, genetic modifications are relatively easy to perform compared to other model organisms (3, 8). Overall, *C. elegans* was a well-suited model organism for this study, serving as an effective, powerful research tool.

In this study, we successfully introduced AHA as a metabolic label into *C. elegans*, allowing for the physical isolation of AHA-incorporated NSPs to analyze the nascent proteome under heat shock. The presence of the azide group in AHA allows for CuAAC, click chemistry reaction with an alkyne for visual detection and enrichment (5). Specifically, a rhodamine-alkyne fluorescent tag was reacted in a CuAAC reaction with AHA for fluorescent confirmation of AHA incorporation into NSPs. Subsequently, a biotin-alkyne tag allowed for strepavidin enrichment to isolate biotinylated proteins. The strong streptavidin-biotin interaction ensures high selectivity and efficient biotin-incorporated protein enrichment (6). Isolated biotin-tagged proteins can then be identified and analyzed with mass spectrometry, since streptavidin's high affinity for biotin improves sensitivity (7).

To metabolically label *C. elegans*, AHA must first be incorporated into *E. coli*, the organism's food source, before subsequent incorporation into *C. elegans* NSPs. This is unlike in mammalian cells, where direct culture in a medium containing the ncAAs allows for labeling (1). *C. elegans'* protective outer cuticle excludes compounds from direct transport into the worm body, and its active digestive system can lead to degradation prior to incorporation. Thus, a specialized delivery method directly transports AHA into the worm's intestine via incorporation into *E. coli* and subsequent consumption to ensure high levels of *C. elegans* labeled proteins (Figure 2) **(**9).

Our method of optimization of AHA labeling in *C. elegans* represented a significant improvement over existing protocol. Our optimized labeling method supported the comprehensive analysis of NSPs in response to heat shock, as confirmed by in-gel fluorescence imaging, assisting a complete systematic understanding of *C. elegans* protein regulation response to stimuli.

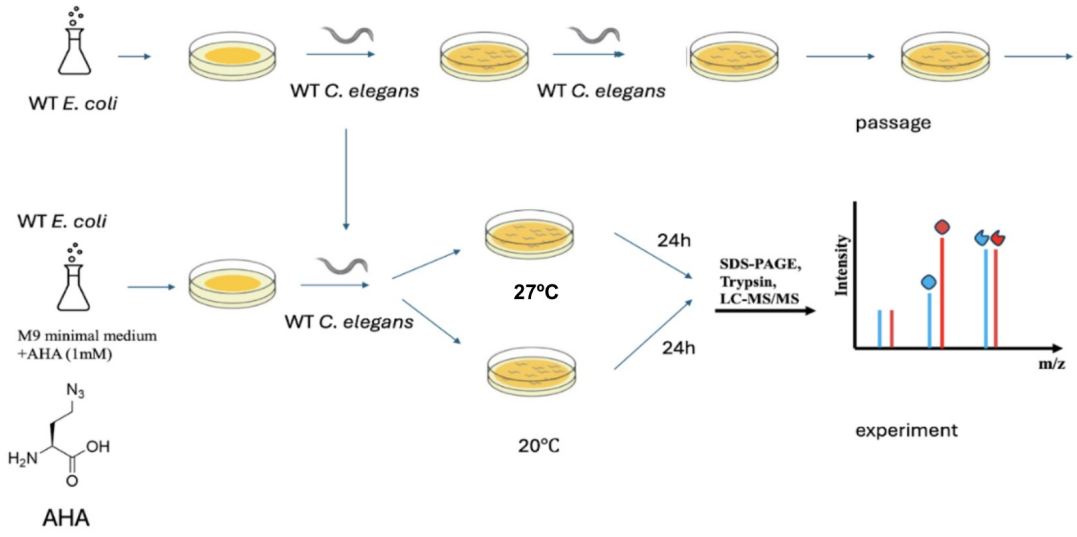

Figure 2. Experiment workflow. Recovery and preparation of *C. elegans,* followed by *E. coli* AHA labeling and subsequent *C. elegans* labeling under optimal environment and heat stress. In-gel fluorescence and mass spectrometry for nascent proteomic analysis.

2. Materials and Methods

2.1 *E. coli* labeling (10)

A starter culture of *E. coli* was prepared by streaking single colonies onto an NGM plate containing LB agar and incubating overnight. One colony was then used to inoculate a new liquid culture, which was grown overnight at 37 °C. The overnight culture was diluted into freshly prepared M9 medium (1× M9 salt solution: 6 g/L Na_2HPO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 g/L KH_2PO_4; supplemented with 20 mM glucose, 2 mM $MgSO_4$, and 100 µM $CaCl_2$), agitated at 37 °C, and grown to an OD_{600} of 0.200.

Cells were pelleted at 5,000 × g for 15 min at 4 °C, washed three times with PBS, and resuspended in freshly prepared M9 minimal medium supplemented with 2.0 mM AHA. After overnight agitation at 37 °C, cells were harvested again by centrifugation (5,000 × g, 15 min, 4 °C), resuspended in fresh M9 minimal medium, and stored at 4 °C.

2.2 *C. elegans* labeling (11)

A culture of *C. elegans* was grown on NGM plates spotted with 200 µL of AHA-labeled OP50 *E. coli* and incubated at either 20 °C or 27 °C for 24 hours. After incubation, worms were rinsed off the plates using 5 mL of M9 buffer per plate, transferred to a 50 mL tube, and pelleted by centrifugation at 2,000 × g for 5 minutes.

2.3 Cell lysis (12)

The pelleted worms were resuspended in 2 mL of lysis buffer (1% SDS, 50 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, pH 7.4) and lysed by sonication. The lysate was then centrifuged at 14,000 × g for 5 minutes, and the supernatant was collected for downstream analysis.

2.4 Rhodamine click chemistry (12)
A fraction of the supernatant was transferred to a 2-mL tube to assess AHA incorporation efficiency. For the click reaction, 100 µM Rho-alkyne was added to AHA-labeled *E. coli* or *C. elegans* lysates, followed by 1 mM TCEP, 100 µM TBTA, and finally 1 mM $CuSO_4$ to initiate the reaction. The mixture was incubated in the dark for 1 hour.

After incubation, proteins were pelleted by centrifugation at 16,000 × g for 5 minutes at 4 °C, and the supernatant was discarded. Pellets were washed twice with ice-cold methanol and air-dried. Protein pellets were resuspended in 1× LDS sample loading buffer (Invitrogen), denatured at 80 °C for 8 minutes, and resolved by SDS-PAGE.

2.5 Biotin click chemistry (13)
Worm lysate supernatant was collected in a 15 mL tube for biotin click chemistry. To the AHA-labeled lysates, 100 µM Bio-alkyne was added and vortexed. Next, 1 mM TCEP and 100 µM TBTA were added, followed by vortexing, and the reaction was initiated with 1 mM $CuSO_4$ with additional vortexing. The mixture was incubated for 1.5 hours.

After the reaction, labeled proteins were precipitated with at least 4 volumes of ice-cold acetone and stored at –20 °C overnight. The samples were thawed, centrifuged at 3,500 × g for 5 minutes at 4 °C, and the supernatant discarded. Pellets were washed twice with ice-cold methanol, centrifuged as before, and air-dried.

The dried pellets were resuspended in 500 µL PBS by vortexing and heated at 75 °C for 8 minutes. The pooled lysate was then diluted with PBS to achieve a final SDS concentration of 0.2%.

2.6 Streptavidin enrichment (for Uncleavable Biotin-Alkyne, 13)
Streptavidin beads were washed five times in a Bio-spin column with 1 mL PBS per wash. The washed beads were then added to the protein sample and rotated for 1.5 hours to allow binding.

Buffers were prepared as follows: Buffer A**:** PBS with 0.2% SDS, Buffer B**:** 6 M urea in PBS with 0.1% SDS, Buffer C**:** 250 mM NH_4HCO_3 in ddH_2O with 0.05% SDS

The sample and beads were transferred to a Bio-spin column, and the flow-through was discarded. Beads were washed six times with 1 mL each of buffers A, B, and C. After washing, beads were transferred to a 1.5 mL tube containing buffer C.

Beads were boiled with 30 µL LDS, 50 mM biotin, and 50 mM DTT. Following this, the samples were treated with 150 mM iodoacetamide, resuspended in 1× LDS sample loading buffer (Invitrogen), denatured at 80 °C for 8 minutes, and resolved by SDS-PAGE.

3. Results

To explore AHA incorporation into NSPs, AHA was first incorporated into the nascent *E. coli* proteome and then this *E.coli* used as a food source for *C. elegans*. The *C. elegans* were able to be metabolically labeled by feeding on AHA-labeled *E. coli*. Experimental groups of *C. elegans* were subjected to a heat shock environment during labeling, affecting protein synthesis and regulation. Fluorescent or affinity tags were used in a click chemistry reaction with

AHA to allow for visualization and enrichment of NSPs (14). Metabolic incorporation was confirmed via in-gel fluorescence analysis, allowing for comparison of the nascent proteomes under 20 ºC optimal growth conditions and 27 ºC heat shock.

E. coli was grown in an M9 medium supplemented with 2.0 mM AHA, supporting *E. coli* NSPs labeling (10). With the azide group from AHA incorporated into NSPs, a CuAAC reaction was made possible in conjugation with rhodamine-alkyne fluorescence (Figure 3a). However, following known protocols for *E. coli* labeling (inducing *E. coli* into AHA medium upon reaching OD of 1.0) revealed low incorporation of AHA into the nascent *E. coli* proteome as demonstrated by SDS-Page in-gel fluorescence scanning (Figure 3b).

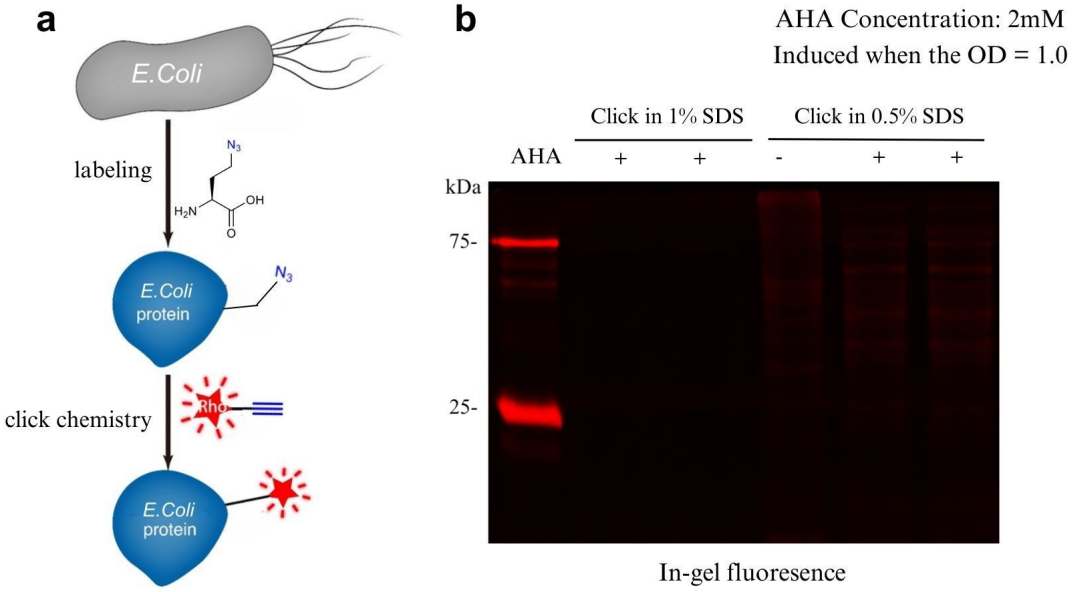

Figure 3. a Labeling workflow for *E. coli* proteins with AHA and subsequent click chemistry with rhodamine fluorescent tag. **b** In-gel visualization of AHA incorporation into *E. coli* after 24-hour incubation with 2 mM AHA. Incorporated AHA was conjugated with a rhodamine-alkyne fluorescent tag in a CuAAC reaction, and NSPs were visualized via in-gel fluorescence, revealing poor labeling efficiency.

Sufficient AHA incorporation is integral to the experiment because it affects the input protein amount for mass spectrometry, thus limiting understanding of the nascent proteome if not enough proteins are biotinylated. Hence, optimizing the AHA labeling efficiency required significant attention to ensure success in later steps.

Upon analyzing different experimental conditions, in-gel fluorescence confirmed that click chemistry signals were strongest in a 0.5% SDS buffer, with most efficient AHA

incorporation found after inducing *E. coli* with an initial OD of 0.2 for 24-hour incubation in AHA-supplemented M9 Medium culture **(**Figure 4a**).** This was supported by the similar protein concentration in the other samples, as determined by the BCA protein assay (Figure 4b), revealing these modified conditions to optimize *E. coli* labeling and click chemistry reactions.

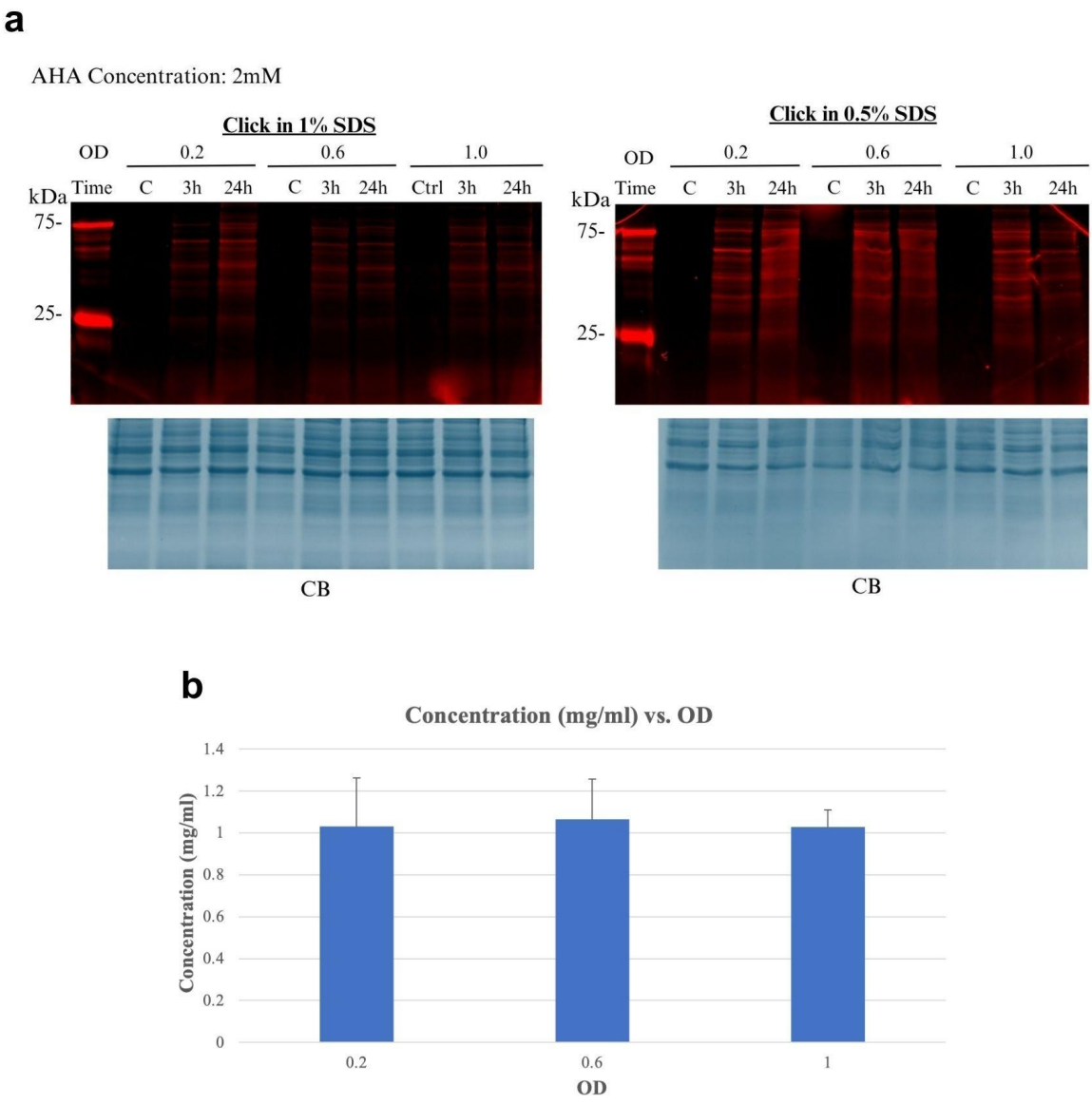

Figure 4. a In-gel visualization of *E. coli* labeling efficiency optimization. SDS-Page gel illustrates the strongest fluorescence signaling from the culture with initial OD 0.2 after 24-hour incubation, and the most effective click chemistry in 0.5% SDS. **b** BCA protein assay reveals statistically similar protein concentration across three initial *E. coli* OD conditions grown over 24 hours.

After identifying the *E. coli* culture with the highest labeling efficiency, this culture was used as the food source for *C. elegans* for a 24-hour feeding period (Figure 5a). Because *E. coli* labeling efficiency correlates directly with labeling efficiency in *C. elegans*, highly labeled bacteria yield higher levels of labeled nematode proteins (15). Thus, selecting the culture induced at an initial OD of 0.2—shown to exhibit optimal labeling based on its strong fluorescence signal—ensured maximal AHA incorporation in *C. elegans*. Following the 24-hour incubation, successful incorporation of AHA into newly synthesized proteins (NSPs) was confirmed by in-gel fluorescence imaging (Figure 5b) (16).

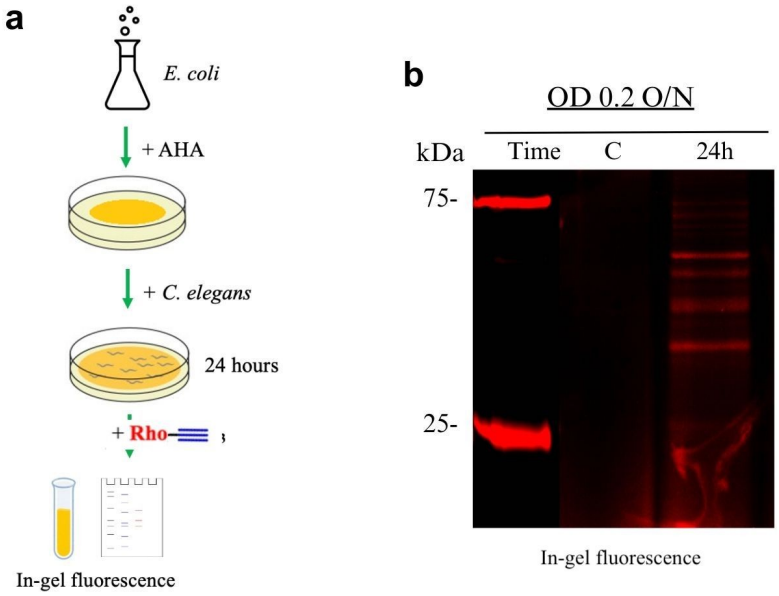

Figure 5. a *C. elegans* labeling workflow. *E. coli*, a food source for *C. elegans*, is primarily labeled with AHA and then fed to *C. elegans*. Rhodamine fluorescent tag undergoes a CuAAC reaction with AHA, followed by in-gel fluorescence, confirming the incorporation of AHA at the methionine site. **b** In-gel fluorescence imaging of AHA incorporation into C. elegans after 24-hour incubation with AHA-labeled *E. coli*. Incorporated AHA was conjugated with rhodamine-alkyne in a CuAAC reaction and visualized through in-gel fluorescence.

Thus, in-gel fluorescence imaging confirmed the successful incorporation of AHA into *E. coli* and *C. elegans* NSPs, prompting heat shock analysis. After optimizing *E. coli* AHA-labeling, subsequent experimental tagging yielded the strongest signaling, enabling a comprehensive comparison of the heat shock and non-heat shock nascent proteomes to analyze *C. elegans'* response to various degrees of environmental stimuli.

C. elegans were incubated with AHA-labeled *E. coli* for 24 hours under 20 ºC or 27ºC conditions. 20 ºC provided the most optimal growth conditions for the model organism, while 27 ºC served as a heat shock. Under this stimulus, *C.*

elegans protein synthesis is expected to differ from regular protein regulation as a result of stress response (17). In-gel fluorescence confirmed the incorporation of AHA into both experimental groups (Figure 6).

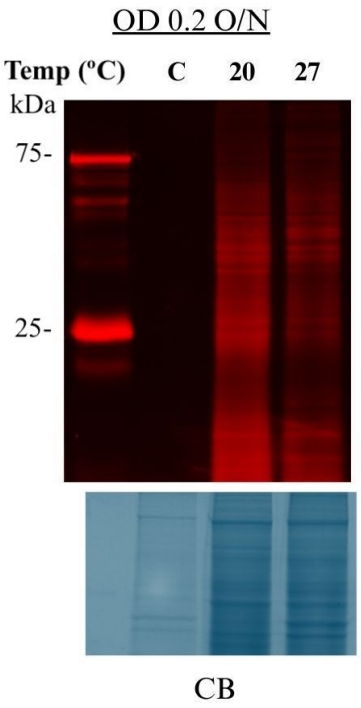

Figure 6. In-gel fluorescence imaging of AHA incorporation into *C.elegans* after 24-hour incubation in 20 °C/27 °C environmental conditions with AHA-labeled *E.coli.*

Finally, subsequent streptavidin enrichment confirmed sufficient AHA-labeled protein in preparation for mass spectrometry analysis of the nascent proteome. Results of such analysis are expected to provide a comprehensive understanding of the *C. elegans'* response to heat shock stimulus. Compared with the input, elution showed successful enrichment with an overall enrichment yield of ~0.5% (Figure 7). With an input of 2mg of protein, the estimated enriched protein concentration is ~16μg, confirming sufficient AHA labeling of *C. elegans* NSPs to enable further downstream analysis.

Experimental results concluded the successful and sufficient incorporation of AHA into the nascent proteome of both *E. coli* and *C. elegans* upon labeling optimization, as confirmed by fluorescence imaging via rhodamine click chemistry. Subsequent conjugation with a biotin affinity tag enabled the further analysis of protein regulation in the organism in response to various biological stimuli through analysis of the nascent proteomes.

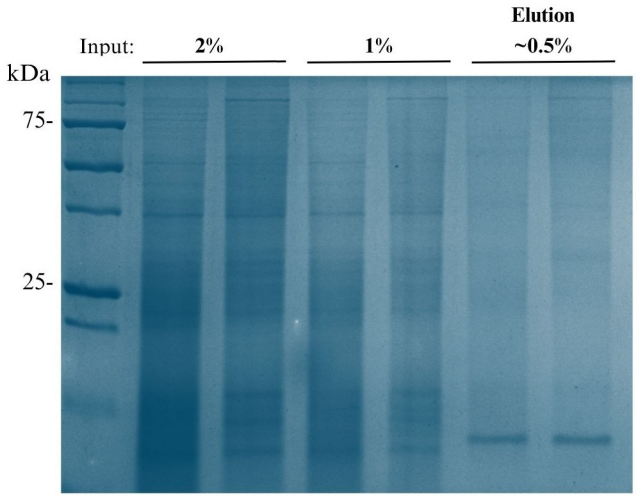

Figure 7. Strepavidin enrichment quality check to estimate enriched protein concentration with regard to the input. Successful enrichment is confirmed with a sufficient yield for mass spectrometry.

4. Discussion

Living organisms respond to environmental stimuli through dynamic protein regulation, undergoing protein synthesis and degradation to maintain cell homeostasis. Nascent proteomics introduces a new method with increased sensitivity and efficiency that allows for the analysis of NSPs within the pre-existing protein population of an organism, which represents an advance over conventional methods that suffer from insufficient sensitivity (1).

In this study, bioorthogonal noncanonical amino acid tagging (BONCAT) was supported by amino acid analog AHA as a metabolic label to be incorporated into NSPs *in vivo* in C. elegans under two temperature conditions, allowing for efficient and non-toxic labeling of the nascent proteome. Heat shock dynamically influences protein regulation pathways, affecting the proteins involved in bioorthogonal reactions, such as click chemistry, compared to those grown under optimal conditions (18). As such, analysis of the nascent proteomes can reveal how *C. elegans* responds to environmental stimuli of varying degrees (19).

AHA functions as a methionine surrogate and becomes incorporated into newly synthesized proteins (NSPs) at methionine positions. Its azide group enables click reactions with alkyne-containing probes, allowing fluorescence imaging and streptavidin-based enrichment. The results of this study demonstrate an improved protocol for optimizing *E. coli* bioorthogonal labeling, achieving robust AHA incorporation in *C. elegans* and revealing differences in NSP profiles through click chemistry. This optimization also ensures that sufficient labeled protein is available for downstream mass spectrometry analysis. Together, these methods provide a powerful framework for dynamically assessing changes in protein abundance in response to diverse environmental stimuli (20).

AHA has been developed and used as a nascent proteomics tool, but AHA-labeling optimization remains a research gap in the field. We modified the existing method to optimize the bioorthogonal labeling efficiency of AHA.

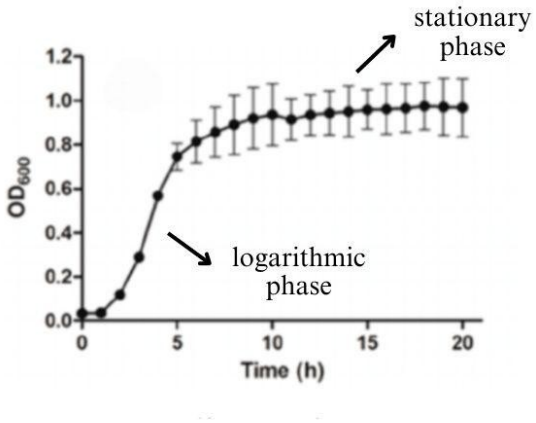

Figure 8. *E. coli* growth curve

We speculated that the *E. coli* OD at the time of induction of 2.0 mM AHA in M9 Medium could affect the AHA labeling efficiency due to the degree of bacterial growth. At OD 1.0; which is the method presented in current protocols; bacterial density is high, with bacteria reaching their stationary phase, where growth begins to stagnate (Figure 8) (20). If then transferred (at OD 1.0) into AHA medium, due to the high bacterial saturation, protein synthesis is stunted, as is the incorporation of AHA into NSPs. Thus, AHA is minimally incorporated into the nascent proteome in the current method as seen in Figure 3b.

However, inducing *E. coli* into the AHA medium while it is still in its logarithmic phase, with exponential bacterial growth, would allow for more protein synthesis in an AHA environment, leading to greater incorporation into NSPs. After 24-hour growth, the three bacterial cultures induced at different initial OD saturations (0.2, 0.6 and 1.0) exhibited similar protein amounts, as indicated by the overlapping error bars in Figure 4b. This suggested that any difference in the strength of the fluorescence signal could be attributed to variations in labeling efficiency across different phases of the E. coli growth curve, rather than to differences in protein amount. We then normalized the protein amount for the click reaction and subsequent in-gel fluorescence, ensuring an equal amount of total protein loaded across all lanes for accurate comparison of labeling efficiency.

Our findings illustrate the strongest fluorescence signal from *E. coli* culture with an initial OD of 0.2 and, interestingly, the weakest labeling with OD 1.0, contradicting known protocol. This supports our hypothesis that AHA labeling efficiency is optimized when *E.*

coli is induced into AHA M9 Medium after reaching an initial OD of 0.2, since the protein concentration was comparable, but the signal was much stronger (Figure 4). At this time, bacteria enter their logarithmic phase, growing exponentially along their growth curve while in an AHA environment, which supports maximum AHA incorporation. This led to a greater concentration of biotinylated proteins, enabling a more comprehensive analysis of the nascent proteome under varying degrees of stimuli in subsequent procedures.

Additionally, the SDS concentration appeared to be an obstacle for effective click chemistry, with fluorescence signaling appearing weak when the reaction was carried out in 1% SDS compared to 0.5% SDS, illustrating that a higher concentration of SDS may affect the click reaction. This is a result of the detergent SDS, which increases solubility and, at higher concentrations, can potentially dissolve proteins (21). At 1% SDS, *C. elegans* proteins become solubilized and denatured, which likely reduces the accessibility of azide groups for click chemistry, leading to a weaker fluorescent signal. Therefore, while the lysis buffer is composed of 1% SDS, diluting the lysate before click chemistry to a lower detergent concentration becomes imperative to ensure the most effective reaction. Under these conditions, proteins are not as impacted by SDS's dissolving capacity as they were under 1% SDS. Similarly, SDS must be diluted to 0.2% for the most efficient biotin tagging during streptavidin enrichment.

After optimization of *E. coli* labeling, AHA is successfully and efficiently incorporated into the nascent proteome under both 20 ºC and 27 ºC growth conditions, as illustrated by the fluorescence imaging. There were visible discernible differences in the protein bands, suggesting that protein regulation did, in fact, differ due to varying degrees of environmental stimuli. Thus, it can be concluded that *C. elegans* does have varying responses to stimuli of different temperatures. Heat stress may have contributed to the induction of protein production or protein misfolding, affecting the level of AHA incorporation and subsequent click chemistry reactions (22). In-gel fluorescence imaging illuminates this dynamic response of *C. elegans* to environmental stimuli.

Upon confirmation of successful labeling, the streptavidin enrichment quality check confirmed sufficient protein concentration that enables the next step of mass spectrometry, allowing for further investigation of the impact that stimuli have on the nascent *C. elegans* proteome through specific identification of the protein population. Mass spectrometry allows for a comprehensive comparison of the identified NSPs under both environmental conditions, with the determination of molecular weight, chemical structure, and protein concentrations in the sample (23). This analysis can reveal the precise effects of heat shock stimulus on the protein regulation response in *C. elegans*, illuminating the details of specific protein types that are synthesized or degraded in response to stress. This, in turn, allows for a greater understanding of *C. elegans's* metabolic pathways.

In conjunction with the mass spectrometry analysis, cell-type selective bioorthogonal labeling in *C. elegans* becomes the next step in further analyzing protein regulation in response

to various biological stimuli. Instead of conjugating the whole worm with an affinity tag to analyze NSPs throughout the entire worm body, protein regulation in specific cells can be analyzed to observe their unique responses to environmental changes. For example, the effects of an environmental change can be investigated in nerve cells and muscle cells selectively and compared to analyze the varying responses a stimulus may elicit across cell types, allowing for a greater understanding of cells' differential responses through protein regulation.

5. Limitations

Since this was an exploratory study, statistical analysis and controls were not as robust. For example, we did not include −AHA, +Met competition, −Cu (for CuAAC), no-alkyne tag control, unlabeled E. coli feeding, acute heat-shock regimen control (e.g., 34 °C for 1 h) vs chronic mild stress, viability/fertility controls, and total-protein loading controls. We also did not report variance, confidence intervals and effect sizes. Tests for normality and homoskedasticity were not performed. Protein loading was normalized for gels, but corrections for worm number, developmental stage, feeding rate, bacterial load, and temperature-induced changes in ingestion were not included. Sensitivity to key parameters (AHA concentration, time course, bacterial strain, worm stage, copper ligand) was not explored; no biological replicates or inter-day repeats were performed.

6. Conclusion

Using an optimized workflow, and exploratory study conditions, the noncanonical amino acid L-azidohomoalanine (AHA) was efficiently incorporated into the nascent proteome of *C. elegans* under both standard and heat-shock conditions. Optimization began with improving *E. coli* metabolic labeling by inducing cultures during the logarithmic growth phase—rather than the stationary phase used in conventional protocols—which increased AHA availability to the nematodes. After ingestion, AHA was incorporated into newly synthesized proteins and selectively tagged through bioorthogonal labeling. Click chemistry subsequently enabled conjugation of rhodamine fluorescent probes and biotin affinity tags for visualization and enrichment. In-gel fluorescence confirmed robust, nontoxic labeling and revealed distinct changes in nascent proteome profiles across different heat-shock intensities. Together, these optimized steps suggest a sensitive and reliable framework for examining dynamic protein regulation in response to environmental stress.

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