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Approaches to studying the increase in the activity and stability of the recombinant aminoacylase from *Escherichia coli* LGE 36

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ABSTRACT

Background:

Optically active amino acids, obtained using the aminoacylase enzyme, play a crucial role in the food, pharmaceutical, and medical industries. Researchers have developed approaches to enhance the activity and stability of the recombinant intracellular aminoacylase from *Escherichia coli* LGE 36. Various stabilizing reagents—dithiothreitol (DTT), glutathione, mercaptoethanol, ascorbic acid, sodium cyanide and sodium sulfide—were studied, with freezing used as a control for comparison.

Results showed that DTT at a concentration of 10^{-3}M facilitated to preserve the activity of the studied enzyme up to 105%. Additionally, the combined use of DTT and Co^{2+} ions tripled the enzyme's activity and improved its stability by 2.5 times. Notably, that the simultaneous action of DTT and Co^{+2} ions also facilitate enzyme activity regeneration. The most effective way to preserve the activity of the recombinant aminoacylase is to freeze the enzyme.

Objective: The objective of the work is to develop approaches to studying the increase in the activity and stability of the recombinant intracellular aminoacylase from *Escherichia coli* LGE 36.

Methods: Recombinant strain-producer of aminoacylase from *Escherichia coli* LGE 36 was used in this study. *E. coli* cells were grown in M9 minimal medium with supplements at 37^0C. Aminoacylase activity was measured at 37°C in a reaction medium with a final volume of 0.2 mL, containing Buffer A (100 mM Na/K-phosphate buffer, pH 7.0), 0.2 mM $CoCl_2$, 40 mM N-acetyl-D,L-methionine, and the required amount of enzyme. One unit of acylase activity was defined as the amount of the enzyme catalyzing the formation of 1 µmol of L-methionie in 1min at 37^0C at pH 7.0.

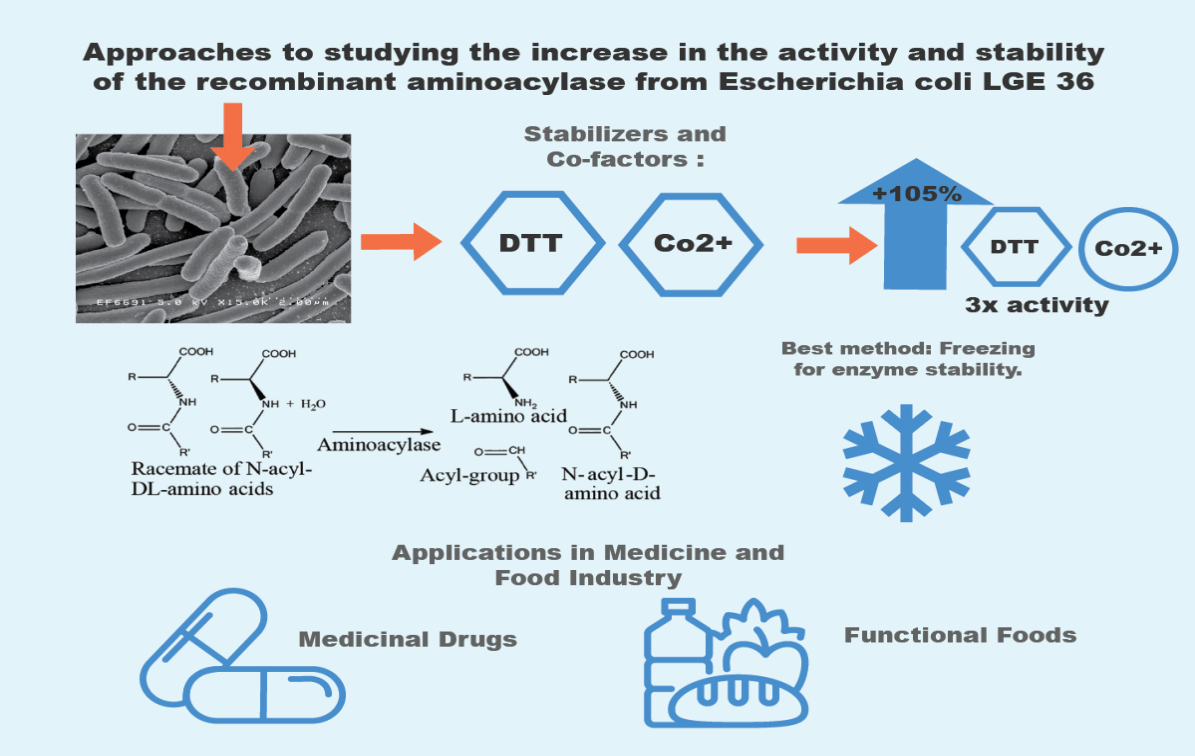
Results: The recombinant intracellular aminoacylase from *Escherichia coli* LGE 36 was obtained using a previously developed method. The effect of various reagents on the stability and activity of the enzyme structure was studied. Results showed that DTT stabilized the recombinant aminoacylase up to 105% compared to the control. The combined use of Co^{2+} ions and DTT increases the enzyme activity threefold and increases the stability by 2.5 times. Additionally, the simultaneous action of DTT and Co^{+2} ions facilitated the regeneration of the enzyme activity. Among the tested methods, freezing proved to be the most effective strategy for preserving the recombinant aminoacylase activity.

Novelty: This study uniquely demonstrates the synergistic effect of dithiothreitol (DTT) and cobalt ions (Co+2) in significantly enhancing both the activity and stability of recombinant intracellular aminoacylase from Escherichia coli LGE 36. This novel approach, including the enzyme's activity regeneration, significantly advances optimizing this industrially important enzyme for producing optically active amino acids.

Conclusion: The obtained data on the properties of the recombinant intracellular aminoacylase make it possible to more fully characterize the biochemical and physiological features of the aminoacylase of *Escherichia coli* 36, an industrially important enzyme. Optically active amino acids obtained by biocatalysis using the recombinant intracellular aminoacylase of *Escherichia coli* LGE 36 can serve as a basis for creating new medicinal drugs and biologically active supplements used in the functional food industry.

Keywords: recombinant aminoacylase, Escherichia coli, enzyme, ion metal, reagents, biologically active supplements, functional food industry.

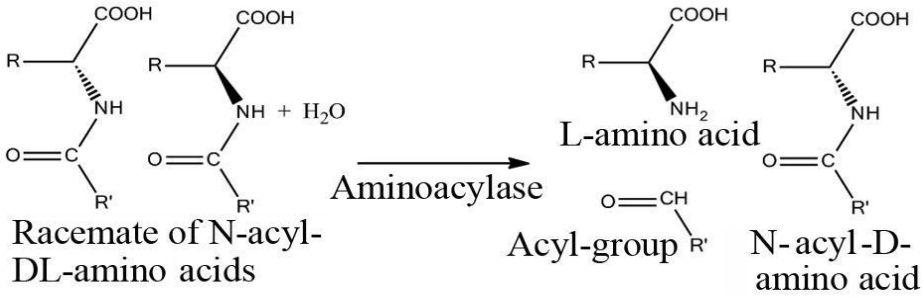
Graphical Abstract



INTRODUCTION

The demand for optically active amino acids is growing every day since their L- and D-forms are widely used in medicine to synthesize several physiologically active compounds, including antibiotics. In particular, D-phenylglycine serves as a crucial raw material for the synthesis of ampicillins and cephalixin. Additionally, amino acids are significant in the food industry as food and feed additives [1]. Traditionally, amino acids are obtained through chemical synthesis, microbiological methods, and biocatalysis. This paper focuses on a biocatalytic approach using the enzyme aminoacylase.

Aminoacylase enzymes (N-acyl-L-amidohydrolase, EC 3.5.1.14) facilitate the enantioselective hydrolysis of N-acyl derivatives of D, L-amino acids. They are widely employed in the industrial production of L- and D-amino acids from racemic mixtures [2]. The stereoselective hydrolysis of N-acylamino acids, catalyzed by aminoacylase, is shown in Scheme 1.



Scheme 1. Separation of amino acids using aminoacylase

Numerous publications have explored the production, immobilization, and characterization of L-aminoacylases from various sources [3–6]. However, despite the widespread occurrence and extensive research, the search for efficient microbial producers of L-aminoacylase remains ongoing. In this regard, studying the characteristics of recombinant intracellular aminoacylase from Escherichia coli LGE 36 is relevant and important. The research also expands knowledge on aminoacylases—enzymes with substantial physiological significance and practical value.

The work aims to develop approaches for enhancing the activity and stability of Escherichia coli LGE 36's recombinant intracellular aminoacylase.

MATERIALS AND METHODS

This study used a recombinant strain of Escherichia coli LGE36 that produces aminoacylase [7]. *E. coli* cells were grown in M9 minimal medium with supplements at 37^0C.

Aminoacylase activity was determined by the modified method of Gade and Brown [8] at a temperature of 370 °C in the reaction mixture containing Buffer A (100 mM Na, K-phosphate buffer, pH 7.0), 0.2 mM CoCl2, 40 mM N-acetyl-D,L-methionine, and the required amount of enzyme. One unit of aminoacylase activity was defined as the amount of the enzyme catalyzing the formation of 1 µmol of L-methionine per min at 370 °C at pH 7.0.

Effects of the reagents on the stability of aminoacylase.

The enzyme was incubated in Buffer A for 15 minutes at 37°C in the presence of various reagents, including dithiothreitol (DTT), glutathione, β-mercaptoethanol, ascorbic acid, Na_2S, and NaCN, at appropriate concentrations. The remaining enzyme activity was assayed by the method described above. The enzyme solution with the appropriate reagents was stored for 7, 14, and 16 days to determine the duration of the recombinant aminoacylase stability.

RESULTS AND DISCUSSION

This work continues studies on the properties of the recombinant intracellular aminoacylase of *E. coli* LGE 36. Previously, the intracellular aminoacylase of *E. coli* LGE 36 was isolated, purified, and partially characterized [9-12]. Despite the obvious significance of studying intracellular aminoacylases, they are inferior in terms of study to other enzymes.

The primary challenge in enzyme isolation arises from their low concentrations in the original preparations, inherent instability, and susceptibility to hydrolysis by accompanying proteases. Enzymes are affected by three main factors: denaturation (due to pH, temperature, or organic solvents), proteolysis, and inactivation of the catalytic center.

Mild conditions were employed to minimize undesirable inactivation during the isolation of recombinant intracellular aminoacylase from E. coli, specifically excluding organic solvents. The serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to prevent proteolysis during biomass collection at a final concentration of 1 mM.

Complete inactivation of the recombinant aminoacylase by p-chloromercuribenzoate (PCMB) suggests the presence of free SH-groups essential for the enzyme activation. These SH-groups are subject to oxidation [12]. To protect the sulfhydryl groups, the following reagents were used as stabilizers for the structure of the recombinant aminoacylase: DTT, glutathione, β-mercapto-ethanol, ascorbic acid, sodium cyanide, and sodium sulfide. Data on the effect of various reagents on the stability of the recombinant aminoacylase are presented in Table 1.

Table 1. Effect of various reagents on the stability of recombinant intracellular aminoacylase of *Escherichia coli* LGE 36.

Reagents	Concentration	Enzyme activity, %		
		0	7	14
		(Days)		
Control*		100	65	42
None (freezing)		100	92	89
Dithiothretol	$2*10^{-4}$M	98	81	55
Dithiothretol	10^{-3}M	105	88	86
Glutathione	10^{-3}M	89	83	56
β -Mercaptoethanol	10^{-3}M	84	78	47
Ascorbic Acid	$5*10^{-3}$M	68	61	-
Na_2S	10^{-3}M	16	-	-
NaCN	10^{-3}M	65	56	-

An enzyme assay was carried out under standard conditions with acetyl-D, L-methionine as a substrate. *The enzyme's activity in the absence of reagents was taken to be 100%.

As can be seen from Table 1, the enzyme loses half of its initial activity when stored at 5 °C for two weeks. DTT has the greatest effect among the stabilizing agents tested, while glutathione, β-mercaptoethanol, ascorbic acid, and sodium cyanide demonstrated weaker stabilization, and sodium sulfide was largely ineffective. DTT effectively preserved enzymatic activity at the same concentration as the other reagents. In the presence of 10^{-3} M DTT, the stability of recombinant aminoacylase was maintained at 105% relative to the control. The most

effective method for preserving recombinant aminoacylase was enzyme freezing.

An investigation into the influence of metal ions on enzyme activity showed that Co^{+2} ions had the maximum activating effect, enhancing the activity of recombinant intracellular aminoacylase from *E. coli* by threefold [12]. The combined effect of Co^{+2} ions and DTT on the activity and stability of the recombinant aminoacylase was studied. The data are presented in Table 2.

Table 2. The effect of Co^{+2} ions and DTT on the enzymatic activity and stability of the recombinant intracellular aminoacylase of *Escherichia coli* LGE 36.

Addition (concentration)*	Enzyme activity, %	
	0	16
	(Days)	
None	100	42
Co^{+2} ($2 * 10^{-4}$M)	280	101
DTT (10^{-3}M)	105	86
Co^{+2} ($2 * 10^{-4}$M) +DTT (10^{-3}M)	310	244
Co^{+2} ($2 * 10^{-4}$M) +DTT ($3 * 10^{-4}$M)	300	241

An enzyme assay was carried out under standard conditions with acetyl-D, L-methionine as a substrate. *The enzyme's activity in the absence of reagents was 100%.

The results presented in Table 2 show that the simultaneous addition of DTT and Co^{+2} ions stimulates the enzyme's activity, while the independent use of DTT shows no stimulating effect. The activity of the recombinant intracellular aminoacylase of *Escherichia coli* increases by 3 times upon the simultaneous addition of Co^{2+} ions at a concentration of $2 * 10^{-4}$M, and the use of DTT at a concentration of 10^{-3}M increases the stability by 2.5 times. While the enzyme is stored, the activity decreased by one-third of the initial activity (Table 1). Co^{+2} ions stimulate preservation of the activity of the recombinant aminoacylase, but no reactivation of the activity lost is observed. Concurrently, the simultaneous addition of Co^{+2} ions and DTT effectively restores the enzymatic activity of the recombinant intracellular aminoacylase of *E. coli.*

When the physicochemical properties of the obtained enzyme were studied, it was found that E. coli LGE 36's recombinant intracellular aminoacylase was similar to those studied in other sources. Complementary to the acylases considered, the enzyme is activated by Co^{+2} ions, sensitive to the action of SH-reagents. The effect of activation by Co^{+2} ions is a characteristic of many aminoacylase enzymes. Still, within this study, the effect is clearly expressed as the activity of the recombinant aminoacylase of *E. coli* LGE 36 increases by 280% upon the addition of Co^{+2} ions [12].

Stabilization of aminoacylases under the influence of sulfhydryl reagents is well-documented for aminoacylases derived from animal and microbial origins. However, reagents affect aminoacylases from different sources in distinct ways. Aminoacylase from *Bacillus stearothermophillus* is stabilized under the influence of glutathione [13]. DTT and mercaptoethanol stabilize the enzyme from *Burkholderia* sp. strain LP5_18B до 80% [3], while aminoacylase *from Thermococcus litoralis preserves enzymatic activity up to 57% in the presence of DTT* 14]. As mentioned, optically active amino acids are used in the food and medical industries. In particular, amino acids such as L-tryptophan, L-lysine, L-methionine, and L-phenylalanine are widely consumed as food and food additives and used in medicine. Therefore, a strong understanding of the structural features of recombinant aminoacylase of *Escherichia coli* LGE 36 [15,16] enables

the production of optically active amino acids using the recombinant enzyme.

Scientific innovation and practical implications: This research innovatively demonstrates the synergistic effect of DTT and Co+2 ions, which triple the activity of recombinant aminoacylase, and increase its stability by 2.5 times. This novel approach, including enzyme regeneration, surpasses traditional methods, facilitating continuous industrial use while reducing optically active amino acid production costs.

These findings substantially improve efficiency and cost-effectiveness in producing crucial amino acids for the pharmaceutical industry, food, and medicine. Enhanced performance facilitates better pharmaceutical drugs and functional food supplements. Freezing serves as a viable preservation method that simplifies handling. This study provides a practical pathway for optimizing this industrial enzyme while fostering sustainable bioprocesses for high-value amino acids, which benefit both healthcare and food technology.

CONCLUSION

Thus, it can be concluded that approaches to studying the effect of various reagents on the stability and activity of the recombinant intracellular aminoacylase from *Escherichia coli* LGE 3 have been developed. It has been shown that DTT has the most substantial stabilizing effect, and when combined with cobalt ions and DTT, there is an increase in activity and stability. Furthermore, enzymatic activity can be regenerated. Optically active amino acids obtained by a biocatalytic method using recombinant intracellular aminoacylase of *Escherichia coli* LGE 36 could be a foundation for developing new biologically active additives in the functional food industry.

Abbreviations: Dithiothreitol DTT; phenylmethylsulfonyl fluoride PMSF; Sodium sulfide Na_2S, Sodium cyanide NaCN.

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