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Some selected properties of the recombinant aminoacylase from *Escherichia coli* LGE 36

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ABSTRACT

Background: Aminoacylase has seen extensive use in the synthesis of L-amino acids. L-amino acids are widely used in the food and medical industries, as well as in healthcare. The effect of metal ions and inhibitors on the enzyme activity of recombinant intracellular aminoacylase of *Escherichia coli* LGE 36 was studied. Acetyl-D,L-methionine and glycyl-L-methionine dipeptide were used as substrates. It has been shown that with the addition of Co^{2+} ions, the acylase activity of the recombinant enzyme towards N-acetyl-D,L-methionine increases 3 times. In the case of glycyl-L-methionine, the peptidase activity of the recombinant aminoacylase of *Escherichia coli* LGE 36 increases more than 40 times. A number of other metal ions studied do not stimulate the hydrolytic activity of the enzyme, moreover some of them even inhibit it. PCMB completely inactivates the recombinant aminoacylase, EDTA reduces enzyme activity by 85%.

Objective: This study aims to investigate how metal ions and inhibitors affect the enzyme activity of recombinant intracellular aminoacylase Escherichia coli LGE 36.

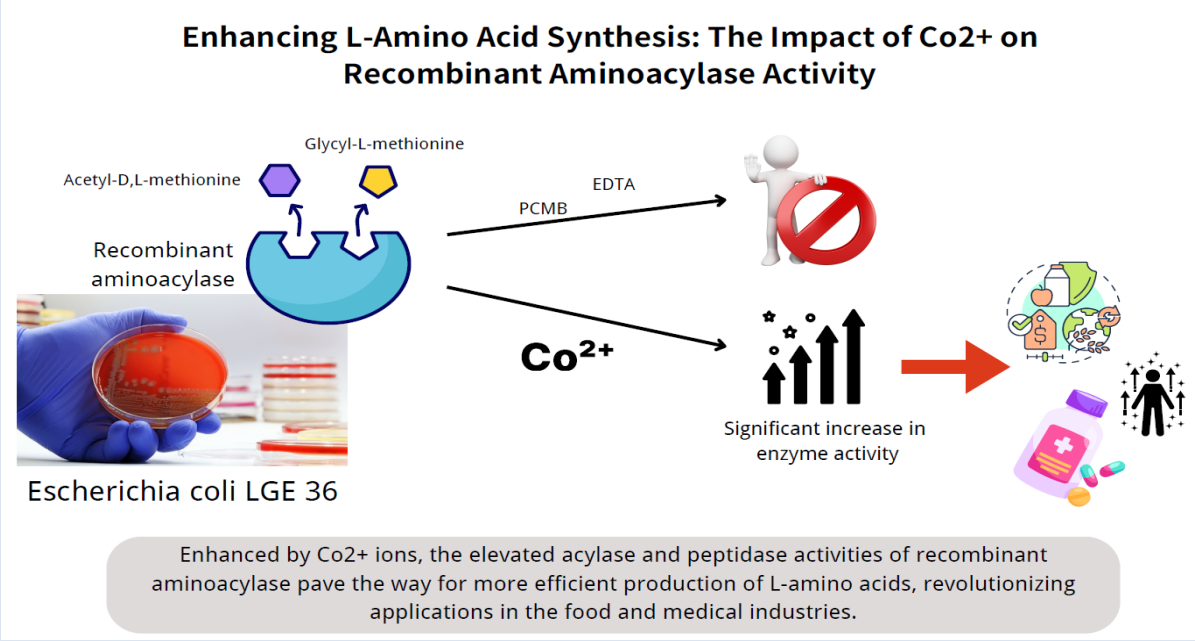
Methods: In this study, a recombinant strain-producer of aminoacylase derived from Escherichia coli LGE 36 was employed. Escherichia coli cells were cultured in M9 minimal medium supplemented at 37°C. To assess aminoacylase activity, assays were conducted at 37°C using a reaction mixture of 0.2 ml final volume comprising 100 mM Na, K-phosphate buffer at pH 7.0, 0.2 mM CoCl2, 40 mM N-acetyl-D, L-methionine, and the enzyme in the appropriate

concentration. Acylase activity was quantified as the amount of enzyme catalyzing the formation of 1µmol of L-methionine within 1 minute at 37°C and pH 7.0.

Results: Some characteristics of the recombinant intracellular aminoacylase from *E. coli* LGE 36 were determined. For investigating the impact of metallic ions and inhibitors, acetylmethionine and glycylmethionine were employed as the substrates.

Conclusion: The present study and the choice of right strategies for functional food products allows optimizing the process for production of optically active amino acids using the recombinant enzyme to obtain food and feed additives.

Keywords: ion, metals, inhibitors, acetylmethionine, glycylmethionine, recombinant aminoacylase from Escherichia coli, enzyme.



INTRODUCTION

Aminoacylase (N-acyl-L-amidohydrolase, EC 3.5.1.14) can be found in microorganisms, animals, and plants. To date, multiple samples of aminoacylases from molds [1], animal tissues [2-3], and bacteria [4-5] have been purified and characterized. Furthermore, genetic precursors for enzymes, including those from Bacillus stearothermophillus and Lactococcus lactis MG 1363, have been introduced into recombinant Escherichia coli cells. Furthermore, genetic precursors for enzymes, including those from Bacillus stearothermophillus and Lactococcus lactis MG 1363, have been introduced into recombinant Escherichia coli cells. Currently, the production of amino acids is a large-scale production, the main consumer of which is the food industry (up to 65%),

as well as the medical industry and healthcare (more than 15%) [7]. Amino acids, such as L-tryptophan, L-lysine, L-methionine, and L-phenylalanine are widely used as food and feed additives; they are also extensively used in medicine.

Despite the widespread distribution of the enzymes and the scale of the search work, in contrast to the relatively well-studied extracellular aminoacylases, intracellular aminoacylases are not sufficiently characterized. In this regard, an urgent task is to study the recombinant intracellular aminoacylase from *Escherichia coli* LGE 36.

The purpose of this work is to study the effect of metal ions and inhibitors on the enzyme activity of recombinant intracellular aminoacylase from *Escherichia coli* LGE 36.

MATERIALS AND METHODS

In this study, a recombinant strain of aminoacylase derived from Escherichia coli LGE 36 [8] was utilized. The cells underwent cultivation in M9 minimal medium supplemented at 37°C. Subsequently, cell disruption was achieved through sonication in 10 mM Na, K-phosphate buffer at pH 7.0, containing 0.2 mM CoCl2 and 1 mM phenylmethylsulfonyl fluoride. Removal of cell debris was carried out via centrifugation, and the resulting supernatant was employed for activity assessment. Aminoacylase activity was quantified using a modified version of the Gade and Brown [3] method, conducted at 37°C in a reaction mixture of 0.2 ml final volume containing 100 mM Na, K-phosphate buffer at pH 7.0, 0.2 mM CoCl2, 40 mM N-acetyl-D, L-methionine, and the requisite enzyme concentration. The unit of acylase activity was determined as the quantity of enzyme catalyzing the production of 1 µmol of L-methionine within 1 minute at 37°C and pH 7.0.

Inhibition of intracellular aminoacylase: PCMB (p-chloromercuribenzoate) was introduced into a solution of the enzyme in phosphate buffer at pH 7.0 with a concentration of 1 mM. The mixture underwent incubation for 60 minutes at 20°C. Afterward, the remaining activity of the substrate N-acetyl-D, L-methionine was evaluated at 37°C. The enzyme's activity without any inhibitor was considered as 100%. Enzyme activity was also tested under similar conditions with the addition of 1 mM EDTA.

Effect of ion metals on the activity of intracellular aminoacylase: Solutions containing divalent metal ions (Co2+, Ca2+, Mg2+, Mn2+, Cd2+, Sn2+, Hg2+) at a concentration of 1 mM were introduced into the enzyme solution in phosphate buffer with pH 7.0. The mixture was then incubated for 60 minutes at room temperature. Activity assessment was performed at 37°C by initiating the reaction with the addition of substrate. The enzyme's activity in the absence of metal ions was considered as 100%. N-acylated amino acids were procured from Sigma-Aldrich. All other reagents were purchased from CIS.

RESULTS AND DISCUSSION

To isolate the enzyme, the recombinant strain of *Escherichia coli* LGE 36 [8] was used, characterized by increased formation of intracellular aminoacylase. The dynamics of growth and development of the aminoacylase activity of the recombinant enzyme was studied. It was found that in a logarithmically growing culture of *E. coli* LGE 36, intracellular aminoacylase exhibited significant enzyme activity at the beginning of the stationary phase of cell growth (Fig. 1). It was shown that the maximum amount of biomass was accumulated within 18-20 hours, and maximum activity was observed at the 18th hour of cultivation of the aminoacylase-

producing strain. Moreover, during the first four hours of cultivation, the pH of the medium dropped by 0.8-0.9 units from the initial pH value; with increased cultivation duration, the pH of the medium increased to a value of 7.6 (Fig. 1). Based on the above, to isolate the enzyme under study, *E. coli* LGE 36 cells were used in the stationary phase at the 18th hour of the culture growth.

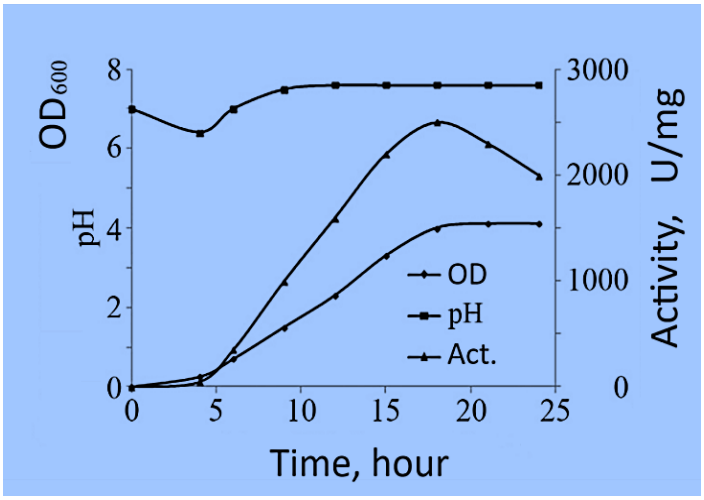


Fig. 1. Dynamics of biomass growth, pH of the medium and activity of intracellular aminoacylase of *Escherichia coli* LGE 36

Next, according to the method developed, the recombinant intracellular aminoacylase was isolated and purified to a homogeneous state using dual ion exchange chromatography on DEAE cellulose [9]. In this work, some characteristics of the recombinant enzyme were determined. To investigate the impact of metals and inhibitors, acetylmethionine and glycylmethionine were employed as the substrates. Various metal ions, ethylenediaminetetraacetate (EDTA), and p-chloromercuribenzoate (PCMB) were introduced into the enzyme assay to assess their impact on activity levels. Additionally, beyond the aforementioned factors, the influence of acyl amino acids on the hydrolysis of glycylmethionine was also examined. The findings presented in Table 1 demonstrate that cobalt ion notably stimulated acylase activity. While manganese, calcium, magnesium, and iron salts at the experimental concentrations showed minimal impact on the reaction, copper, zinc, and nickel salts exhibited inhibitory effects. In the instance of glycylmethionine, manganese notably enhanced the hydrolytic activity, with the degree of activation by these ions significantly surpassing that observed for acylase activity. This was in addition to the results seen with cobalt. Additionally, the inhibitory effect of zinc, copper, and nickel salts was manifested at a much lower level. Marked inhibition by a metal binder EDTA and sulfhydryl reagent PCMB was observed in all substrates tested.

The active site of aminoacylase has been demonstrated to be associated with metal ions, which are essential for maintaining proper activity levels [10]. In conjunction with the aforementioned experiment, the stimulation of recombinant aminoacylase from Escherichia coli LGE 36 by cobalt ions was evident (Table 1). Thus, the influence of ion concentration was investigated using acetyl and glycyl derivatives of methionine as experimental compounds. Under the experimental conditions, the optimal concentration was found to be at 10^-3M or 10^-4M. This resulted in a three-fold activation in acylase activity and over forty-fold stimulation of peptidase activity. . These results are shown in Figure 2.

Table 1. Effect of metals and inhibitors on the enzyme activity of recombinant aminoacylase of *Escherichia coli coli* LGE 36

Additions	Acetyl Methionine	Glycyl Methionine
None	100	100
Cu	0	74
Mg	88	86
Ca	90	138
Zn	6	78
Mn	110	1100
Fe	103	128
Co	209	3800
Ni	11	97
EDTA	15	48
PCMB	0	7

The assay was conducted under standard conditions with a final concentration of additions set at 10^-3M. The activity levels of each respective substrate without any other additions were standardized to 100.

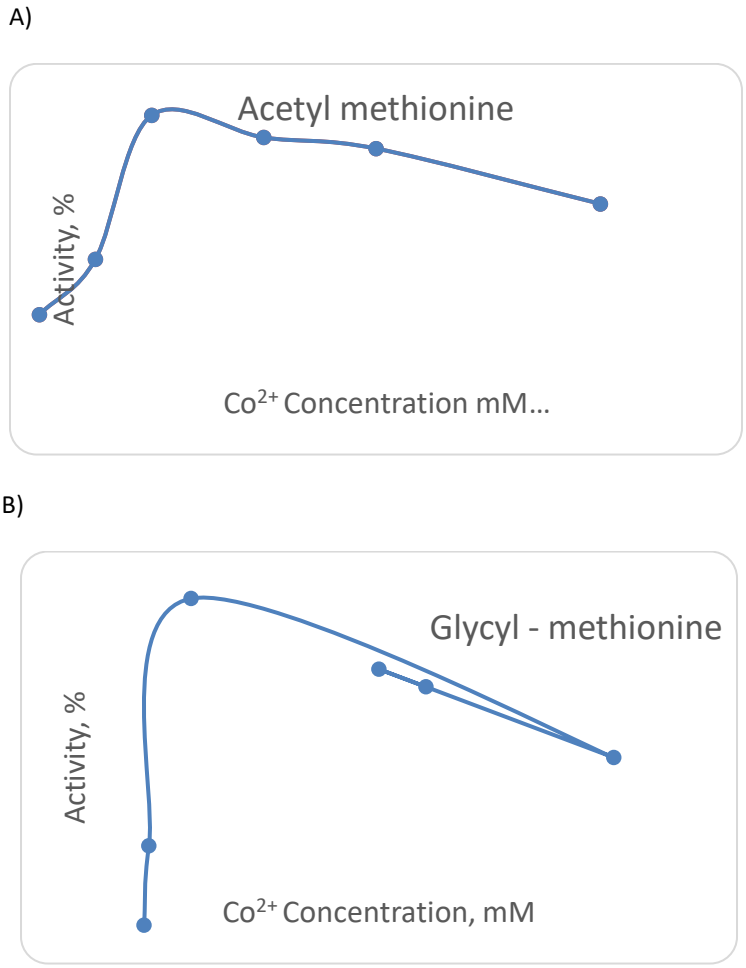


Fig. 2. Investigating the impact of cobalt concentration on the enzymatic hydrolysis of acetyl-methionine (a) and glycyl-methionine (b) of recombinant aminoacylase of *Escherichia coli* LGE 36.

The experiment was conducted under standard conditions with varying levels of cobalt chloride present. The present study also shows that the recombinant aminoacylase is similar to other acylases such acylases fromhog kidneys and mold acylases in some ways. These common characteristics include specific responses elicited by cobalt ions. The inhibition observed upon addition of EDTA and PCMB suggests that both metal ions and sulfhydryl groups play pivotal roles in the action of this recombinant enzyme. Interestingly, aminoacylase of *E. coli* was shown to catalyze the hydrolysis of dipeptide at pH 7.0Research on the characteristics of the recombinant intracellular aminoacylase from E. coli LGE 36 has unveiled that its hydrolytic activity towards glycyl-methionine, acting as a peptidase, surpassed its aminoacylase activity. This dipeptidase activity was proposed to arise due to the substitution of the acyl moiety of the amino acid by the N-terminal amino acid of the dipeptide, acting as a target for hydrophobic interactions within active site regions [11]. Additionally, dipeptidases have multiple similarities with aminoacylases. Some examples of this include metal content, their subunit molecular weight and the requirements of the amino acid composition. The amino acid sequences in each of the N-terminal regions are notably similar [12], suggesting that dipeptides may indeed serve as appropriate substrates for aminoacylases. Studies have previously found that dipeptidase activity can be found in mammalian aminoacylases [11,13] and some bacterial aminoacylases [4]. In this investigation, we did not endeavor to discern the distinctions between acylase activity and peptidase action, despite observing some differences in the influence of metal ions on both activities. For instance, while cobalt ions notably stimulated both peptidase and acylase activities, manganese ions similarly enhanced peptidase activity but not as significantly as cobalt ions did for acylase activity. Moreover, the inhibitory effects of copper and other metals on acylase activity were not as pronounced as initially anticipated. There are currently many sdudies devoted to the production of both native and immobilized aminoacylases and the study of their properties [14-18]. Despite this, the search for an aminoacylase catalyst continues.

Earlier, due to the practical orientation of our work, to obtain optically active amino acids, we studied poorly purified enzyme preparations of aminoacylases and cells of *E.coli* with aminoacylase activity. We have developed a method for obtaining a new highly selective and stable biocatalyst (BC) of L-aminoacylase [Yepremyan H., Author's Certificate No. 1623908, 1991]. BC of aminoacylase are cells of the recombinant strain of *E.coli* LGE 36 with aminoacylase activity immobilized in a silicic acid gel. BC of aminoacylase is used for producing L-met, L-ala, L-val and other optically active amino acids. As is known, L-methionine is one of essential amino acids found in proteins and is the body's main source of sulfur. The absence of methionine or its insufficient amount in the body leads to metabolic disturbance, liver diseases and anemia. Methionine and its derivatives are widely used as food and feed additives, as well as in medicine [19]. 14Undoubtedly, knowledge of the mechanism of action of the recombinant enzyme and the choice of right strategies [19, 20] make it possible to optimize the process of producing optically active amino acids using the recombinant enzyme.

CONCLUSION

As described above, some selected properties of the recombinant intracellular aminoacylase from *Escherichia coli* LGE36 have been studied. The results obtained allow to understand the mechanism of aminocylase action in depth. We intend to continue investigating the occurrence and enzyme features of acylase activity in

recombinant strain of *E. coli* and in various organisms to understand the biological significance of acylase activity. In the future, this work will contribute to creation of new medicinal preparations and biologically active additives used in the functional food industry based on L-met and other optically active amino acids.

Abbreviations*:* EDTA - ethylenediaminetetraacetate, PCMB - p-chloromercuribenzoate

Competing interests: The author declares no competing interests.

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