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Pseudomonas aeruginosa Expressing *Vitreoscilla* Hemoglobin Shows Increased Production of L-Lysine α-Oxidase: an Enzyme used in Cancer Therapy

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Abstract

L-lysine α-oxidase (LO) is one of a few microbial enzymes with therapeutic potential in certain cancers. The enzyme has been determined in several bacteria and fungi. Its production is mainly regulated by carbon sources and oxygen. . Thus, the aim of this study was to determine the nutritional requirements and effect of oxygen concentration on the production of LO in *P. aeruginosa*, and in their recombinants using a highly efficient oxygen uptake system, the *Vitreoscilla* hemoglobin. This study concerns the effect of a higher oxygen uptake provided by a recombinant system, the *Vitreoscilla* hemoglobin, on the production of LO in *Pseudomonas aeruginosa*. The results showed that the recombinant bacterium expressing *Vitreoscilla* hemoglobin gene (*vgb*) had distinct L-lysine activity from the host strain under both carbon catabolite repression and no repression conditions. In a rich medium supplemented with glucose, the recombinant strain showed 20-40 % higher L-lysine activity than the host strain. This difference was even more significant in the medium with no glucose supplement, where the recombinant strain showed almost 2-fold higher enzyme activity throughout the incubation. The results sometime were contradictory in terms of the effect of carbon source (mainly glucose) and oxygen on the production of this enzyme.

Keywords: *Vitreoscilla* hemoglobin, *Psedomonas aeruginosa*, L-lysine α-oxidase

Vitreoscilla Hemoglobini İçeren *Pseudomonas aeruginosa*'da Kanser Tedavisinde Kullanılan Bir Enzim Olan L-Lisin α-Oksidaz Üretimi Arttırılması

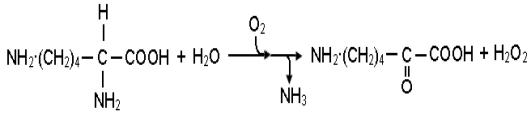
Özet

L-lizin birçok bakteri ve mantarda tespit edilmiştir. Enzimin üretimi esas olarak karbon kaynakları ve oksijen ile düzenlenmektedir. Bu nedenle, bu çalışmanın amacı, *Pseudomonas aeruginosa*'daki LO üretimindeki besin gereksinimlerini ve oksijen konsantrasyonunun etkisini, yüksek verimli bir oksijen alım sistemi olan *Vitreoscilla* hemoglobin kullanan rekombinantlarda a-oksidaz (LO), bazı kanserlerde terapötik potansiyeli olan birkaç mikrobiyolojik enzimden biridir. Enzim belirlemektir. Bu çalışma, bir rekombinant sistem olan Vitreoscilla hemoglobini tarafından *P. aeruginosa*'da LO'nin üretimine yüksek oksijen alımının etkisi ile ilgilidir. Sonuçta, *Vitreoscilla* hemoglobin genini (*vgb*) eksprese eden rekombinant bakterinin, hem karbon katabolit baskısı hem de baskı koşulu olmaksızın rekombinat suştan farklı L-lizin aktivitesine sahip olduğunu göstermektedir. Glikozla zenginleştirilmiş zengin bir ortamda, rekombinant suş, yabanıl bakteriden % 20-40 daha fazla L-lizin aktivitesi göstermiştir. Bu fark, rekombinant suşun, üretim boyunca neredeyse 2 kat daha yüksek enzim aktivitesi gösterdiği glikoz takviyesi olmayan ortamda ise daha da anlamlıydı. Sonuç olarak, bu enzimin üretiminde karbon kaynağının (esas olarak glikoz) ve oksijenin etkisi açısından bir çelişkide görülmektedir.

Anahtar Kelimeler: *Vitreoscilla* hemoglobin, *Psedomonas aeruginosa*, L-lysine α-oxida

1. Introduction

L-Lysine α-oxidase (LO) (1.4.3.14) as one of the potent antitumor enzymes was isolated and purified from *Trichoderma viride Y-244-2* for the first time in Japan [1]. L-lysine α-oxidase (LO) belongs to the group of oxidases of L-amino acids. The enzyme catalyzes oxidative deamination of L-lysine yielding hydrogen peroxide, ammonia, and the corresponding keto acid, α-keto-ε-aminocaproic acid: [2]



LO has a molecular weight of approximately 116,000 and 2 mol of FAD/mol of enzyme; it consists of two subunits of identical molecular weight (about 56,000): 1 mol of FAD is bound/subunit [3-4]. The determination of many biological nitrogen compounds has relied on monitoring the ammonia released from enzymatic reactions [1, 5-6]. This reaction is frequently monitored by means of amperometric detectors. One of the most important applications of this enzyme is its use as the chemotherapeutic agent in certain kinds of cancers where it is used to "starve" cancer cells of an essential amino acid, L-lysine. The chemotoxic effect of the enzymes is a result of such restriction that causes growth inhibition of neoplastic cells. Since the middle of the last century some enzymes have been of special interest for investigators due to the possibility of their application in medicine, particularly in oncology. Essential amino acids, including l-lysine, cannot be synthesized in the body [7-8]. Thus, enzymes capable of cleaving these amino acids are promising for investigation aimed at their application in oncology [3-4, 7]. The study of LO biological property shows that it exhibits a sufficient level of cytotoxicity toward tumor cells. LO shows antileukemic activity and antimethastatic effect *in vivo* at rather low efficient doses [3, 7]. Glucose is an essential nutrient in industrial microbiological systems that produce lysine [9]. The used of organic chemicals such as solvent hexane for the release of periplasmic enzymes. Might be advantageous as they are costs effective, and cause selective permeabilization of the outer cell wall barriers allowing large-scale preparation of periplasmic enzymes in a relatively pure form. Gram-negative bacteria, *P. aeruginosa*, could regulate the production of LO, an enzyme expressed by an oxygen-regulated gene. Thus, the aim of this study was to determine the nutritional requirements and effect of oxygen concentration on the production of LO in *P. aeruginosa*, and in their recombinant use a highly efficient oxygen uptake system, the *Vitreoscilla* hemoglobin [10]. In this connection, maintaining an adequate supply of oxygen to aerobically growing cell cultures is a central problem in a variety of bioprocesses involving long-scale growth of microorganisms. This may be particularly severe when recombinant cells are involved.

In this paper, different spectrophotometric determination of lysine based on the detection of ammonia is developed.

2. Materials and Methods

2.1. Chemicals

Trichloroacetic acid (TCA), Nessler reagent chemicals (HgI_2, KI and NaOH), hexane were purchased from Sigma Chemicals Co. All other chemicals used were of analytical grade. Culture medium and stock solution of carbohydrates were autoclaved separately at 120 ºC for 25 min.

1.2. Bacterial strains and cultivation

The bacteria used in this study was *P. aeruginosa* (USDA B771) obtained from the USDA culture collection in Peoria, IL. The transposon-mediated *vgb* transferred recombinant strain of *P. aeruginosa*, named PaJC was described previously [11]. The growth media used for LO production were Luria-Bertani (LB) (MILLER 1972), and semi-synthetic (MMY and MM) medium [12] both at pH 7.0. LB contained (L^{-1}) 10 g peptone, 5 g yeast extract and 10 g NaCl. MMY was (L^{-1}) 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.5 g KCl, 1 g K_2HPO_4, 0.5 g yeast extract and 1.5 g l-lysine as the nitrogen source. MM was no added yeast extract. Cells were maintained on LB agar plates at 4 ºC with transfers at monthly intervals. A 1/100 inoculate of overnight cultures grown in

LB was made in 20 ml LB in 125 ml Erlenmeyer flasks. Inoculate in flasks were grown for 24 h at 37 ◦C in a 250 rpm water-bath.

2.3. Permeabilization with solvent

Cells cultivated for LO production were harvested by centrifugation (10,000 rpm for 5 min) at 18 ºC temperature, washed once with 0.05M potassium phosphate (KPi) buffer (pH 7.6), and resuspended to A_{600} = 5.0 in the same buffer containing organic solvent which was the %2 optimal concentration of hexane for LO release. The suspensions were incubated at room temperature for 1 h, briefly vortexing for every 10 min. Tube caps were left open for 5 min in order to evaporate volatile upper phase prior to analysis of LO activity in the cell-free aqueous phase. Cell suspensions were also made in 0.05M KPi with no solvents. In both types of controls, cells were first suspended in KPi for 30 min.

2.4. Enzyme assay

Apart from amperometric methods, optical monitoring of the ammonia released by LO has been proposed. Ammonia participates in further reactions to form spectrophotometric active derivatives. The enzyme activity was measured by the method of Wriston (1970), use the Nesslerization reaction. This method uses the determination of ammonia liberated from LO in the enzyme reaction by the Nessler reaction. Reaction was started by adding 0.5 ml permeabilized cell suspension from aqueous phase into the 1.5 ml 0.01M l-lysine prepared in 0.05M KPi buffer, pH 7.6 and incubated for 1 hour at 37 ºC. The reaction was stopped by the addition of 0.15 ml 1.5 M TCA. The reaction mixture was centrifuged at room temperature (10,000 rpm for 5 min) to remove the precipitate and the ammonia released in the supernatant was determined colorimetrically (A_{480}) by adding 0.25 ml Nessler reagent into tubes containing 0.5 ml supernatant and 1.75 ml dH_2O. The content in the tubes was vortexed and incubated at room temperature for 10 min, and the A_{480} values were reads against the blanks that received TCA before the extract addition. One LO unit (U) is defined as the amount of enzyme that liberates 1 µmol of ammonia per min at 37 ºC. Specific activity is expressed as units per milligram of protein released. The ammonia concentration produced in the reaction was determined on the basis of a standard curve obtained with ammonium sulfate as the standard. The limit of detection of ammonia by this method was about 10µM [13].

Permeabilization was carried out at roomtemperature on cell suspensions made in 50mM KPi, containing hexane to 2%. Cells were harvested and suspended in respective buffers to equal A_{600} = 5.0. Each value is the average of three independent experiments with error *bars indicating STDEVs* (σ_{n-1}).

2.5. Protein determination

Total protein was determined colorimetrically [14], using bovine serum albumin as the standard.

3.Results

Since LO acts selectively on l-lysine, virtually not affecting the rate of oxidative deamination of other natural amino acids, structural analogs of l-lysine [3].

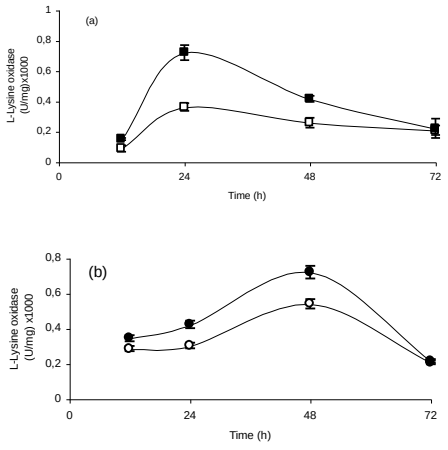
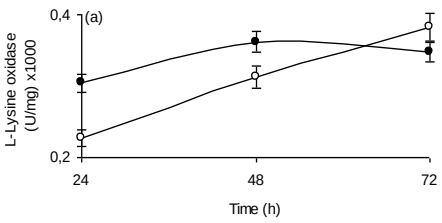


Figure 1. L-lysine α-oxidase activity from *P. aeruginosa* (o) and its *vgb* recombinant (■) grown in LB (a) and LBG (b) medium.

The production of LO, *P. aeruginosa* and *Vitreoscilla* hemoglobin (VHb) expressing recombinant grown under different culture

conditions was investigated. Bacteria were grown in rich or semi-synthetic media with carbon source to determine both the extent of catabolite repression reported for this enzyme. The effect of efficient oxygen uptake system is the VHb, on LO production. Bacteria were grown in different media with or without carbohydrate supplement. Both *P. aeruginosa* and *PaJC* showed distinct profiles in terms of LO levels. The production of LO by bacteria grown in LB or LB with glucose is summarized in Figure 1. LO production was studied in cells harvested following 72 h cultivation. In *P. aeruginosa*, however, as strain PaJC had a superior LO level to its parental strain. The oxygen uptake rates of PaJC were higher than the non-*vgb*-bearing strains [15]. When compared with their parental strains, much higher oxygen uptake rate of PaJC [11] might contribute to this difference, as LO is an enzyme repressed under high oxygen. *P. aeruginosa* and its recombinants grown in LB medium with 1% glucose showed a different pattern of enzyme activity to that of cells grown in LB with no glucose. In general, the bacteria in MMY and MM medium showed lower enzyme activity than their counterpart in LB medium. *P. aeruginosa* and its *vgb*$^+$ strain (PaJC) grown in these medium with 1% glucose had more than 1.5-fold higher LO activity than in LB medium. The effect of glucose on LO activity in *P. aeruginosa* and its recombinant PaJC grown in MMY and MM was opposite to that no glucose MMY and MM medium. At 1% and %0.1 concentrations, however, glucose had opposite results on enzyme production in *P. aeruginosa* and PaJC (Figure 2 and Figure.3).

In general the level of LO in bacteria grown in glucose-supplemented MMY medium was significantly lower than in glucose-supplemented MM medium. The presence of VHb, however, had a differential effect in these two bacteria grown in glucose medium.



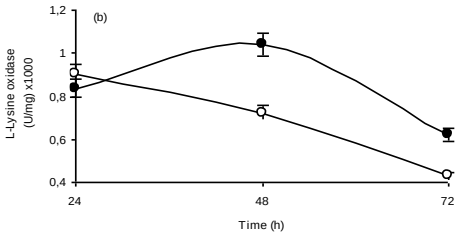
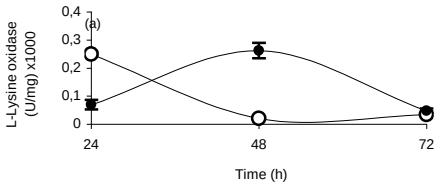


Figure 2. L-lysine α-oxidase release from *P. aeruginosa* (o) and its recombinant (■) KPi/hexane aqueous phase system in MMY a) 1% and b) 0.1% glucose medium.

A comparison of KPi/hexane aqueous phase



system on LO release (determined as specific activity) from *P. aeruginosa* is given in Figure. 2-3. Cells were suspended in 50mM KPi/hexane at pH 7.6 to A_{600} = 5.0 for permeabilization extract preparation.

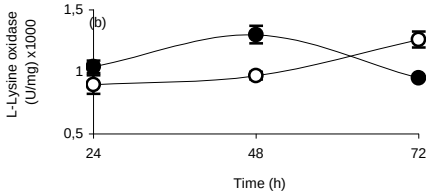


Figure 3. L-lysine α-oxidase release from *P. aeruginosa* (o) and its recombinant (■) KPi/hexane aqueous phase system in MM a) 1% and b) 0.1% glucose medium.

Although similar in medium pH when they were grown in LB and LB with 1% glucose, *P. aeruginosa* and PaJC showed approximate pH values. They were grown in MMY and MMY with 1% glucose, *P. aeruginosa* and PaJC showed distinct pH values. The average medium pH in LB or LB with 1% glucose was 8.60 ± 0.04 for *P. aeruginosa* and its recombinants 8.11 ± 0.04 for PaJC strain. The pH values in semi-synthetic MMY medium supplemented with 0.1% and 1% glucose may also reduce.

4.Discussion

LO is not produced in cultures under anoxia. We showed that the presence of highly efficient oxygen uptake system, VHb, affects differently the production of LO in three distinctly related medium. The system used here is based on the membrane permeabilization of cells by an aqueous salt/organic solvent phase system; KPi/ hexane. The aqueous phase was analyzed for enzyme activity and total protein content after separation of organic phase, which required a simple evaporation of highly water immiscible hexane. The membrane permeabilization system (50mM KPi/ 2 % hexane) used here was highly effective on specific release of LO from bacteria. Furthermore, at concentrations ≥0.5 M, KPi heavily interfered with the absorbance (A_{480}) reading as it caused a heavy turbidity by reacting with Nessler reaction reagents. The hydrophobicity characteristics of solvents are commonly indicated as log *P*, where *P* is the partition coefficient of the solvent between 1-octanol and water. In general, solvents with low log *P* values are regarded as poorer enzyme releasers than solvents with high log *P* values (*P* > 2.0). Although hexane with the highest log *P* value (log *P* = 3.5) was the most efficient solvent for LO release. Among the solvents with log *P* values >2.0, hexane is the smallest molecule which makes it penetrate more efficiently into the outer cell membrane, resulting disorganization of the outer membrane enough to cause the secretion of LO. Due to low affinity for water, hexane can be removed from aqueous phase by a simple evaporation causing almost no contamination or denaturation. Furthermore, contrary to mostly toxic effect of surfactants and other membrane destabilizes on cells, hexane is non-toxic and can be used as suitable medium for microbial processes, such as in whole-cell catalysis. To determine both the expression level and the reaction rate a constant of the enzyme from these sources, the enzyme has to be purified is our next goal.

5.References

1. Saurina, J., Herna'ndez-Cassou, S., Alegret, S., and Fa'bregas, E. (1999). Determination of lysine in pharmaceutical samples containing endogenous ammonium ions by using a lysine oxidase biosensor based on an all-solid-state potentiometric ammonium electrode. *Biosens. Bioelect*, 14: 67-75.

2. Saurina J., Herna´ndez-Cassou S., Alegret S., and Fa`bregas E. (1999). Amperometric determination of lysine using a lysine oxidase biosensor based on rigid-conducting composites. *Biosens. Bioelect.*, 14: 211-220.

3. Treshalina, H.M., Lukasheva, E.V., Sedakova, L.A., Firsova, G.A., Guerassimova, G.K., Gogichaeva, N.V., and Berezov, T.T. (2000). anticancer enzyme L-lysine α-oxidase. *Appl. Biochem. Biotechnol*, 88: 267-273.

4. Kusakabe, H., Kodama, K., Kuninaka, A., Yoshino, H., Misono, H., and Soda, K. (1980). A new antitumor enzyme, L-lysine α-oxidase from *Trichoderma viride*, Purification and enzymological properties. *J. Biol. Chem.*, 255: 976-981.

5. Galaris, D., and Evangelou, A. (2002). The role of oxidative stress in mechanisms of metal-induced carcinogenesis. *Crit. Rev. Oncol. Hematol.*, 42: 93-103.

6. Li, W., Nugent, M. A., Zhao, Y., Chau, A. N., Li, S. J., Chou, I., Liu, G., and Kagan, H. M. (2003). Lysyl oxidase oxidizes basic fibroblast growth factor and inactivates its mitogenic potential. *J. Cell. Biochem.*, 88: 152-164.

7. Lukasheva, E.V., and Berezov, T.T. (2002). L-Lysine-α-Oxidase: Physicochemical and biological properties. *Biochem.*, 67: 1152-1158.

8. Murthy, S. N., and Janardanasarma, M. K. (1999). Identification of L-amino acid/ L-lysine α-amino oxidase in mouse brain. *Molec. Cell. Biochem.*, 197: 13-23.

9. Almuaibed, A. M., and Townshend, A. (1997). Flow injection amperometric and chemiluminescence individual and simultaneous determination of lysine and glucose with **i**mmobilized lysine oxidase and glucose oxidase. *Anal. Chim. Acta*, 338: 149-154.

10. Geckil, H., Gencer, S., and Uckun, M. (2004). *Vitreoscilla* hemoglobin expressing *Enterobacter* differently to carbon catabolite and oxygen repression for production of L-asparaginase, an enzyme used in cancer therapy. *Enzy. Microb. Technol.*, 35: 182-189.

11. Chung, J. W., Webster, D. A., Pagilla, K. R., and Stark, B. C. (2001). Chromosomal integration of the *Vitreoscilla* hemoglobin gene in *Burkholderia* and *Pseudomonas* for the purpose of producing stable engineered strains with enhanced bioremediating ability. *J. Indust. Microbiol.Biotechnol.*, 27: 27-33.

12. Mukherjee, J., Majumdar, S., and Scheper, T. (2000). Studies on nutritional and oxygen requirements for production of L-asparaginase by *Enterobacter aerogenes*. *Appl. Microbiol. Biotech.*, 53: 180-184.

13. Saurina, J., Herna'ndez-Cassou, S., Fa'bregas, E., and Alegret, S. (1998). Potentiometric biosensor for lysine analysis based on a chemically immobilized lysine oxidase membrane. *Analy. Chim. Acta*, 371: 49-56.

14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.

15. Geckil, H., Gencer, S., Kahraman, H., and Erenler, S.O. (2003). Genetic engineering of *Enterobacter aerogenes* with *Vitreoscilla* hemoglobin gene: cell growth, survival and antioxidant enzyme status under oxidative stress. *Res. Microbiol.*, 154: 425-431.