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Purification of Soluble Membrane-Bound *Ambystoma mexicanum* Epidermal Lipoxygenase from *E. coli* and Its Growth Effect on Human Fetal Foreskin Fibroblast

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Abstract
Lipoxygenases are non-heme iron-containing lipid dioxygenases enzymes that catalyze the hydroperoxidation of lipids. The Mexican axolotl (*Ambystoma mexicanum*) is a prominent source of the enzyme with a regeneration capacity in limbs. It has been shown that transfected human osteosarcoma and keratinocyte cells with epidermal lipoxygenase (LOXe) have an increased rate of cell migration. In the present study, LOXe, a peripheral membrane protein, was produced in *Escherichia coli.* The enzyme was purified using different detergents, anionic solutions, and gel filtration chromatography. Kinetic assay of the enzyme activity was carried out by the spectroscopy method using arachidonic acid as a substrate. Finally, the enzyme was characterized and its growth effect on human fibroblast cells was examined by MTT viability assay. Enzyme kinetic parameters including K_m of 90.4 µM and V_{max} of 2.63 IU were determined for LOXe. The enzyme with 0.1 nM end concentration promoted the growth of 5000 cells/well human fibroblast cells up to 11% ($P < 0.01$). In the present study, we introduce an *E. coli* expression system to produce an excessive amount of soluble LOXe and the efficient purification method to provide a soluble and active form of LOXe that is effective in stimulating human fibroblast cell proliferation.

Keywords Peripheral membrane protein · Lipoxygenase activity · Purification · Leukotriene · Wound healing

Abbreviations

HETE	Hydroxyeicosatetraenoic acid
HFFF-PI6	Human fibroblast cell line
IU	International unit
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LOX	Lipoxygenase
NP-40	4-Nonylphenyl-polyethylene glycol
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1 Introduction

Lipoxygenases (LOXs; EC1.13.11.12) are a family of lipid peroxidation enzymes that play a major role in the metabolism of unsaturated fatty acids in plants and animals [1]. In plants, these enzymes catalyze dioxygenation of linoleic acid and linolenic acid to hydroperoxy derivatives, which is thought to be involved in the aging and plant responses to injuries [1]. Moreover, in animal tissues, the main substrate for lipoxygenases is arachidonic acid that is deoxygenated to precursors participating in inflammatory processes, cell membrane maturation, cancer metastasis, angiogenesis, and osteoporosis [2]. This enzyme family not only plays pivotal roles in the biosynthesis of lipid hormones such as leukotrienes and lipoxins but also catalyzes the peroxidation of polyunsaturated fatty acids. In mammals, LOX products and their metabolites are potent lipid mediators that provoke diverse biological responses like inflammatory reactions. One of these metabolites is hydroxyeicosatetraenoic acid (HETE), a potent growth-promoting factor that facilitates the proliferation of fibroblast cells and subsequently promotes wound healing, which is involved in inflammation [3, 4].

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Some species of amphibians such as frogs and salamanders have the limb regeneration ability through the formation of the blastemal [5]. Mexican Axolotl (*Ambystoma mexicanum*) is a prominent example of such species due to its ability to regenerate limbs and scar-free healing [6, 7]. The effect of epidermal lipoxygenase (LOXe) from *Ambystoma mexicanum* on human tissue regeneration and wound healing has been investigated in vitro [8]. Recently, it has been shown that the transfection of human osteosarcoma and keratinocyte cells with LOXe increases the rate of cell migration that can be effective in improving wound healing [9]. To date, this enzyme has not been purified from *Escherichia coli* (*E. coli)*-based producing systems. Furthermore, the enzyme effects on human fibroblast cells as dermis main cells have not so far been studied.

Therefore, the aims of our study were the production of *Ambystoma mexicanum* LOXe in *E. coli* and evaluation of different methods of LOXe purification through determining enzyme activity and its function on the growth of human fibroblast cell line.

2 Materials and Methods

2.1 Materials

The bacterial strain (*E. coli* BL21 (DE3) and Origami-B) and human fibroblast cell line (HFFF-PI6, NCBI: C-170) were supplied from Pasteur Institute (Iran). Arachidonic acid and ampicillin were provided by Sigma (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) and protein weight marker were purchased from Thermo Scientific™ (Germany). The DNase and RNase enzymes were purchased from Sinaclon (Iran). All other chemicals were purchased from Merck (Germany).

2.2 LOXe Production in *E. coli*

Cloning and production of the LOXe gene from *Ambystoma mexicanum* in *E. coli* strains (BL21 (DE3) and Origami-B) were performed successfully based on a developed method in our previous study [9]. The culture medium was incubated at different temperatures (15, 20, and 37 °C) for 19 h. The bacteria cells were harvested by centrifugation at 15,000×*g* for 20 min at 4 °C and stored at − 20 °C.

2.3 Bacterial Cell Disruption and Protein Extraction

The bacterial cell pellet (300 mg) was resuspended in 30 mL of lysis buffer (100 mM Tris/HCl, 5 mM EDTA, pH 7.4, and lysozyme 1 mg/mL) [10]. The DNase (0.14 mg/mL) and RNase (0.14 mg/mL) enzymes were added to the lysate [11]. The suspension was incubated for 24 h at 4 °C. After centrifugation, the isolated pellet was washed twice with 10 mL of first washing buffer (Tris/HCl 100 mM, NaCl 50 mM, pH 7.4) and centrifuged at 11,000×*g* for 10 min. Then the isolated pellet was washed with 10 mL of second washing buffer (1% Triton X-114 in first washing buffer) and centrifuged at 11,000×*g* for 10 min. Afterward, the separated cell pellet was resuspended in 10 mL of another buffer (washing buffer with 1 M NaCl) and incubated for 24 h at 4 °C. The suspension was centrifuged at 11,000×*g* for 10 min at 4 °C. The protein solubility and final extraction from cell pellets were examined with different substances as a solvent. These substances were 50, 100, 200, 500 mM and 1 M NaCl; 100, 200, and 500 mM KCl; 1%, 2%, and 5% NP40; 3%, 6%, and 10% ethanol; 3%, 6%, and 10% methanol; 0.5, 1, 2, and 4 M urea; 0.5%, 1%, and 3% Tween 20; 0.5%, 1%, and 3% Tween 80; 0.5%, 1%, and 3% Triton X-114. 1 mg of the washed pellet was dissolved in 1 mL of each mentioned solvents and incubated at 4 °C for 24 h. After centrifugation at 15,000×*g* for 1 min at 4 °C, the supernatants and pellets were separated. Then dissolved fractions including supernatants and pellets were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4 Final Purification of LOXe by Gel Filtration Chromatography

Solubilized LOXe from bacterial crude was loaded on a Hiload Sephadex 75 column that had been equilibrated with 10 mM Tris/HCl, 250 mM NaCl, pH 7.4. Protein concentration was finally determined by Bradford assay [12].

2.5 Enzyme Activity Assay

Enzyme activity was assessed by a UV–Vis spectrophotometer (Shimadzu 1240 UV–Vis spectrophotometer). Arachidonic acid (diluted with ethanol to 3 mM) was used as a substrate. The conjugated diene 5-hydroperoxyeicosatetraenoic acid (HPETE) and HETE formation catalyzed by lipoxygenase were measured at 234 nm after 20 min at 20 °C with $\varepsilon = 25{,}000\ M^{-1}\ CM^{-1}$ (Fig. 1) [13]. All measurements were performed in triplicates.

2.6 Enzyme Characterization of Purified LOXe

The pH stability for LOXe was examined by performing the reaction in different pH ranging from 4.0 to 10.0 for 20 min at 20 °C. The optimum temperature for the enzyme activity was determined by evaluating the enzymatic reaction at different temperatures (4, 10, 15, 20, 25, 30, and 37 °C) for 20 min [13, 14]. The reaction buffer included 50 mM Tris/HCl, 60 µM arachidonic acid, 0.3 nM pure enzyme with a total volume of 500 µL.

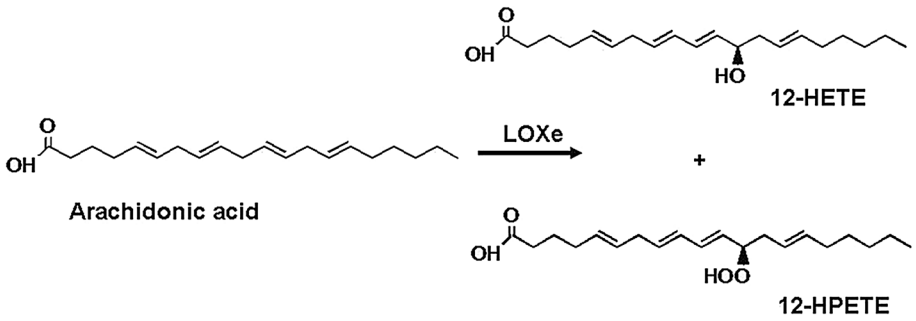

Fig. 1 Lipoxygenase Reaction. Arachidonic acid as a substrate is converted by LOXe to 5-hydroperoxyeicosatetraenoic acid (HPETE) and hydroxyeicosatetraenoic acid (HETE). The conjugated diene HPETE and HETE formation catalyzed by lipoxygenase were measured at 234 nm after 20 min at 20 °C

2.7 Enzyme Kinetics

The K_m value of LOXe was determined using different concentrations of arachidonic acid (6, 12, 30, 45, and 60 µM) as substrate and 50 mM Tris/HCl (pH 7.0) as the reaction buffer. All measurements were performed in triplicates.

2.8 Cell Proliferation Assay

The effect of the LOXe enzyme on human fibroblast cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [14]. HFFF-PI6 cells were propagated and maintained in RPMI medium (Gibco, USA) supplemented with 1% fetal bovine serum (FBS, Gibco, Australia), penicillin (100 U/mL) and streptomycin (100 U/mL) (Gibco BRL, Life technology) in a humidified atmosphere at 37 °C with 5% CO_2. Briefly, 5000 cells/well were seeded in a 96-well plate and incubated at 37 °C under 5% CO_2 and cultivated until the confluency of 70%. The cells were then treated with different concentrations of purified LOXe enzyme (0.003, 0.03, 0.06, 0.1, 0.2, and 0.3 nM). After 48 h, 10 µL MTT (5 mg/mL) was added to each well and the cells were incubated for 3–4 h. Mitochondrial dehydrogenases of viable cells convert MTT into formazan crystals. The culture medium was then discarded and 150 µL dimethyl sulfoxide (DMSO) was added to each well to dissolve the insoluble formazan, and the absorbance was measured at 570 nm in a microplate reader (BioTek Hybrid Multi-Mode Reader, USA) to determine the number of viable cells.

2.9 Statistical Analysis

Statistical analysis was performed with SPSS 20. The One-Way ANOVA (analysis of variance) test was performed to measure the statistical differences among groups. As necessary, data were marked with $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$ which were considered to be statistically significant.

3 Results

3.1 The Optimum Temperature for Protein Production

SDS-PAGE pattern of proteins extracted from *E. coli* strains (BL21 (DE3) and Origami-B) containing the *Ambystoma mexicanum* LOXe gene showed the formation of a new protein band with a molecular mass of about 70 kDa in comparison to non-recombinant bacteria. The maximum production level of this protein band was related to bacteria grown at 20 °C, implying the optimum temperature for LOXe production (Fig. 2).

3.2 The Effective Method for LOXe Extraction from *E. coli*

Based on SDS-PAGE analysis, among various extraction methods, NaCl solution (Tris/HCl 10 mM, NaCl 0.25, 0.5,

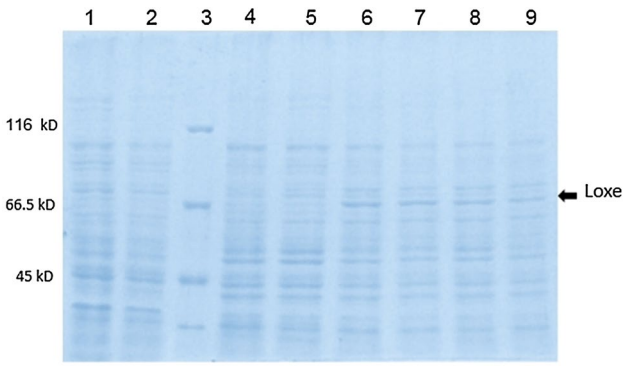

Fig. 2 The SDS-PAGE analysis of LOXe production in different *E. coli* strains and temperatures. (1) Origami B-LOXe before induction by IPTG, (2) BL21(DE3)-LOXe before induction with IPTG, (3) molecular weight marker (kDa), (4) Origami B-LOXe after induction with IPTG in 15 °C, lane (5) BL21(DE3)-LOXe after induction with IPTG in 15 °C, (6) Origami B-LOXe after induction with IPTG in 20 °C, (7) BL21(DE3)-LOXe after induction with IPTG in 20 °C, (8) Origami B-LOXe after induction with IPTG in 37 °C, (9) BL21(DE3)-LOXe after induction with IPTG in 37 °C

and 1 M, pH 7.4) was selected as the most effective method for proteins extraction from the peripheral membrane. This finding indicated that LOXe has been produced as a peripheral membrane protein in *E. coli*. After bacterial cell wall disruption, cell membrane isolation and three times washing of the cell membrane components by washing buffer the protein solubilization and extraction was performed by 250 mM NaCl solution (Fig. 3).

3.3 Purification of LOXe

After enzyme extraction from cell pellets and dissolving in 10 mM Tris/HCl, 250 mM NaCl and pH 7.4, it was purified by gel filtration chromatography on HiLoad Sephadex column in isocratic mode to characterize the LOXe. The comparison of SDS-PAGE results before and after gel filtration chromatography showed that most solubilized protein impurities were located in 35–45 kDa bands (Fig. 3). In addition, the electrophoretic pattern of eluted fractions obtained from the Sephadex column indicated that the prominent single bands with a molecular weight of about 71.6 kDa in an eluted fraction at 44th and 45th minutes were related to the LOXe protein with the maximum purity. This result represents that other impurities have been removed by gel filtration. A summary of purification is given in Table 1. An overall 2.5-fold purification was achieved with a yield of 77%.

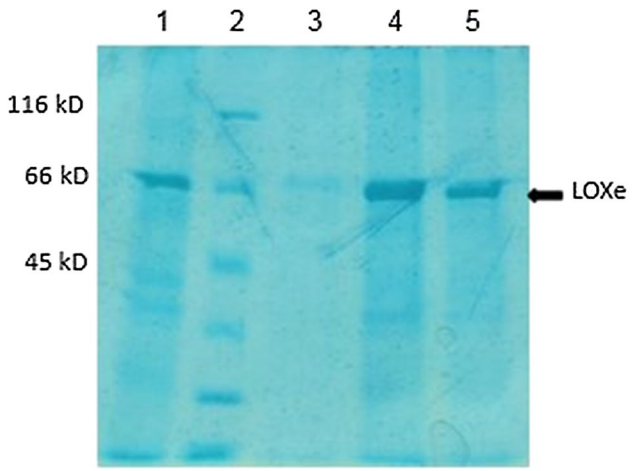

Fig. 3 The solubility optimization of LOXe. The SDS-PAGE analysis of protein extraction and solubilization in optimum condition by 250 and 500 mM NaCl solutions. (1) Final cellular pellet after protein extraction, (2) molecular weight marker (kDa), (3) supernatant of cell wall lysis by lysozyme treatment, (4) supernatant of protein solubilization by 500 mM NaCl solution, (5) supernatant of solubilization by 250 mM NaCl solution

3.4 Effects of pH and Temperature on Enzyme Activity

The optimum pH value from 4.0 to 10.0 and a temperature range from 4 to 37 °C for the enzymatic reaction of LOXe were determined (Fig. 4a, b). According to our data, the highest activity for recombinant LOXe was obtained at pH 7.0 and 15 °C.

3.5 Kinetic Analysis of Purified LOXe

Kinetic analysis of LOXe activity was performed using arachidonic acid as a substrate at the optimum pH of 7.0 and a temperature of 15 °C for 20 min. The K_m and V_{max} values were found to be 90.4 µM and 2.63 IU, respectively (Fig. 4c).

3.6 Effect of Purified LOXe on Human Fibroblast Cells

The results of the MTT assay indicated *LOXe* effects on the proliferation of HFFF-PI6 cells. Our results showed that the treatment of HFFF-PI6 cells with 0.003, 0.03, 0.06, 0.1, 0.2 and 0.3 nM of *LOXe* resulted in increased proliferation to 114, 118, 121, 125, 127 and 148% of control, respectively ($P < 0.05$, Fig. 5).

Table 1 Purification of Axolotl epidermal lipoxygenase (LOXe) from *Escherichia coli* production system

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	100	nd	nd	nd	1
Pellet after washing I	21	nd	nd	nd	nd
Pellet after washing II	17	nd	nd	nd	nd
Pellet after washing III	12	nd	nd	n.d	nd
Solubilization I	9.7	29,200	3010	100	1
Solubilization II	5	26,800	5360	91.7	1.8
Gel filtration	3	22,500	7500	77	2.5

nd not detected

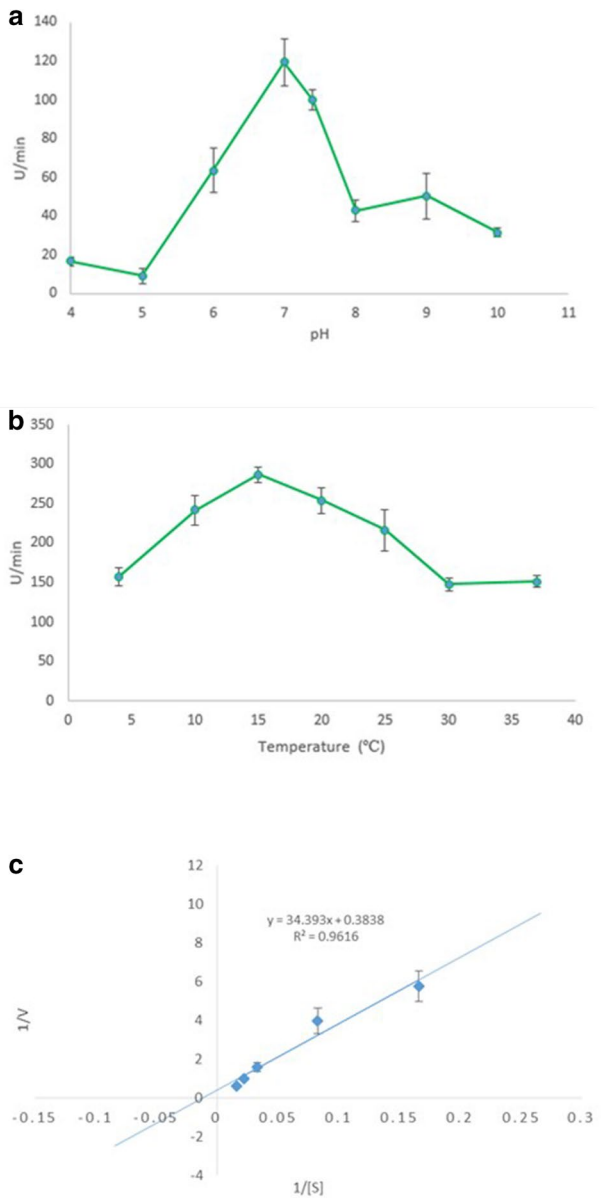

Fig. 4 The pH, temperature and kinetic analysis of purified LOXe on enzyme activity. **a** Effect of pH on the activity of purified LOXe. **b** Effect of temperature on the activity of purified LOXe. **c** Line weaver Burk Plot of the effect of substrate concentration on the activity of LOXe. Different concentrations of arachidonic acid were used as substrate and the enzyme activity was plotted to 1/V and 1/S. All measurements were performed in triplicates

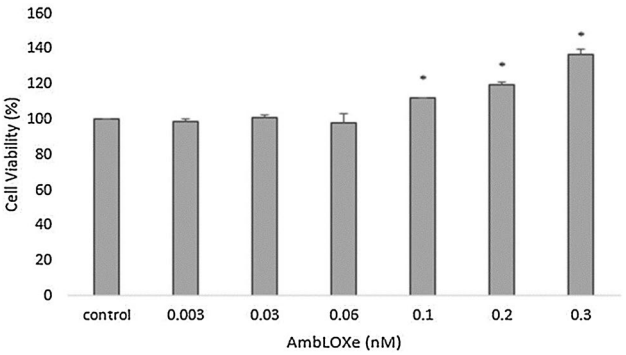

Fig. 5 Enzyme's effect on human fibroblast cells. The enzyme incubation for 48 h with LOXe increased cell proliferation. Asterisk stands for statistically significant between treated and control cells, $P < 0.05$

4 Discussion

LOXe belongs to non-heme iron-containing lipoxygenases that catalyze the peroxidation of polyunsaturated fatty acids. It is an important enzyme in the biosynthesis of active oxylipins which acts as a signaling molecule involved in various biological processes [15]. LOX isozymes play important roles in wound healing, inflammation control, cell migration, and blood clot formation through metabolites obtained from LOX reactions [16]. Mexican axolotl (*Ambystoma mexicanum*) is a prominent organism to have a capacity for regeneration of its limbs [17]. In a study, it has been postulated that the epidermal lipoxygenase of this amphibian plays a vital role in limb regeneration. Investigating the distribution pattern of the enzyme in the tissues revealed the maximum amount of LOX in the epidermal cells of the isolated limbs of Mexican axolotl. It has been shown that transfected human osteosarcoma and keratinocyte cells with LOXe increased the rate of cell migration [8].

To date, various strategies have been used for the purification of lipoxygenases from plants, humans, and animals. Likshmi et al. purified lipoxygenase in soybeans by precipitation, using polyethylene glycol, followed by aqueous two-phase extraction [18]. Lipoxygenase was also isolated from sweet corn by extraction with 0.2 M Tris/HCI, afterward fractionation with 40–60% saturated ammonium sulfate and dialysis [19]. In another study, two different lipoxygenases with molecular weights of 93 kDa (LOX1) and 45 kDa (LOX2) was purified from *Lasiodiplodia theobromae* using size-exclusion (Sephadex G100) and anion exchange (DEAE-cellulose) chromatography [20]. 5-Lipoxygenase in human peripheral blood leukocytes was purified from homogenates by ammonium sulfate fractionation and gel filtration chromatography [14]. Human 15-lipoxygenase was purified by strong anion exchanger chromatography Mono Q column [21]. In previous studies, the purification of lipoxygenase has been mainly reported based on the cytosolic protein purification strategy.

In the present study, for the first time, we showed that LOXe is produced as a peripheral membrane protein. LOXeprotein contains two domains, an N-terminal PLAT-domain (Polycystin-1, Lipoxygenase, Alpha-Toxin) and a C-terminal Lipoxygenase-domain, containing the catalytically active non-heme iron and the substrate-binding cavity [15]. The

PLAT-domain is peripheral and the Lipoxygenase-domain is cytosolic. In our previous study, the cloning of the LOXe gene in *E. coli* (BL21) was performed successfully [9]. In the present study, the extraction, solubilization, and purification of the enzyme were successfully performed up to 77% yield by gel filtration chromatography with a 250 mM NaCl solution. Moreover, we achieved a 2.5-fold purification of the LOXe recombinant protein.

Various values of K_m and V_{max} have been reported in different kinetic studies for human lipoxygenases. The K_m and V_{max} values for human 5-LOX were determined 22.3 μM and 2.56 IU, respectively [13]. The K_m of 63.1 μM and V_{max} of 5.3 IU were detected for human 15-LOX [21]. The K_m value of 15 μM was reported for human 12-LOX [22]. In the present study, the K_m and V_{max} for LOXe were found to be 90.4 μM and 2.63 IU that are relatively high in comparison with kinetic parameters of human lipoxygenases. Our results showed that the optimum pH and temperature for the activity of the recombinant LOXe are 7.0 and 15 °C respectively which are similar to human 15-LOX.

An average increase of 11% in the growth of fibroblast cells exposed to LOXe ($P < 0.01$) demonstrated the positive effect of LOXe on promoting cell growth, in agreement with our results, it has been shown that an increased activity of human 5-LOX induced by co-cultivation with platelets resulted in the stimulation of human dermal fibroblast proliferation. This finding indicated that skin cells can directly respond to LOX enzymatic activity [22]. Furthermore, the results of the MTT assay showed the induction of fibroblast cell proliferation in different concentrations of the purified enzyme, suggesting its effectiveness in the human wound healing process. However, further studies, especially on animal models, could be suggested for understanding the exact molecular mechanisms of enzyme action.

5 Conclusion

The method of protein extraction indicated that LOXe was produced in *E. coli* as a peripheral membrane protein. The results of the MTT assay showed that the purified enzyme increased human fibroblast cell proliferation significantly in a concentration-dependent manner suggesting its potential in the human wound healing process.

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Author Contributions Maryam Mashkouli: designed and performed the experiments, analyzed data and wrote the manuscript. Mahmoud Aghaei: designed, performed experiments and analyzed data. Mohammad Reza Mofid: designed and performed the experiments, analyzed data and wrote the manuscript.

Data Availability The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Compliance with Ethical Standards

Conflict of interest All authors declare that there is no conflict of interest.

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