

Article

Cytotoxic effects of L.asparaginase Purified from *Lactobacillus reuteri* on breast cancer cell lines

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ABSTRACT

L-asparagine is an enzyme widely identified as one of the most potential anti-cancerous drugs. This enzyme is poorly bioavailable, so its pharmaceutical uses are limited as an anticancer. L-asparaginase is an important chemotherapeutic drug used to treat a variety of malignant tumors. Lactic acid bacteria may exhibit high L-asparaginase productivity, efficiency, and anticancer activity against breast cancer cell lines. The L-asparaginase was extracted and purified in multiple processes, including precipitation with (NH₄)₂SO₄ (70%), DEAE-cellulose ion exchanger chromatography, and Sephadex G 150 filtration. The specific activity was 40 U/mg, and the purification fold was 25.6, yielding 51%. The cytotoxic effect of the enzyme on treated cells was dose-dependent, with an IC₅₀ value of 4.305 IU/ml.

Keywords: Cytotoxic effects, L-asparagine, *Lactobacillus reuteri*, breast cancer cell line

INTRODUCTION

Breast cancer (BC) is the most frequent cancer in women, the second most common cancer worldwide, and the second primary cause of cancer-related deaths¹. For over 50 years, drugs with enzymatic activity have been used in clinical medicine. Some enzymes that irreversibly destroy certain vital amino acids are being developed as immune modulator agents and antitumor therapeutics, such as lacto peroxidase, L- glutaminase and Arginin deaminase^{2,3}. The first bacterial enzyme introduced into routine clinical practice was L-asparaginase. Depending on their inducibility, l-asparaginase can be subdivided into two types: cellular localization, affinity to the substrate, and quaternary structure⁴. The first type of enzyme is present in the cytoplasm, expressed constitutively with a lower affinity to l-asparagine and hydrolyze l-glutamine. The Second type of enzyme is present in

the periplasm with a lower affinity to l-glutamine and a higher affinity to l-asparagine⁵. Neoplastic cells cannot synthesize asparagine due to the absence or shortage of L-asparagine synthetases and are dependent on an exogenous supply of this amino acid from the bloodstream⁶. The anticancer effect of L-asparaginase is based on its ability to hydrolyze L-asparagine to L-aspartate and ammonia. The exposure of tumor cells, mainly leukemic cells, to L-asparaginase leads to disturbance of protein synthesis and cancer cell starvation, resulting in their death⁷. The most commonly used bacterial strains for probiotic purposes are the lactic acid bacteria⁸. The enzyme has been shown to have cytotoxic action and is currently used to treat acute lymphoblastic leukemia⁹. So, this study aimed to investigate the cytotoxic activity of purified L. asparaginase isolated from *Lactobacillus reuteri* in the breast cancer cell line.

MATERIALS AND METHODS

Thirty isolates of *Lactobacillus reuteri* isolated from mother milk were plated in de Mann–Rogosa–Sharpe (MRS) agar and incubated at 37 °C for 48 h anaerobically. The distinct colonies based on their colony morphology from each plate were further purified by subsequent sub-culturing in the same media 3–4 times to ensure their purity. To screen LAB, the isolates were subjected to Gram's staining reaction, endospore staining, oxidase, and catalase activities and identified by API 50¹⁰.

Preparation of crude L-asparaginase

Crude L-asparaginase purified from *L. reuteri* LHMZS isolate no.24 was prepared according to the protocol mentioned by¹¹, with some modifications as follows. Afterward, the culture was propagated in the same broth at 37 °C for 48 h under anaerobic conditions. The cells were sedimented by centrifugation at 10000 rpm for 15 min at 4 °C, and then the pH 7 supernatant aliquots were stored at 4 °C until assayed.

Purification of L-asparaginase

Precipitation of enzyme by ammonium sulfate

The enzyme was precipitated with different concentrations of (NH₄)₂SO₄ (20, 30, 40, 50, 60, 70, 80, and 90 percent) saturation under chilled conditions, and the activity of the enzyme, protein concentration and specific activity were determined at each concentration¹¹.

Purification of L-asparaginase by Ion exchange chromatography

DEAE-cellulose column was prepared according to Whitaker and Bernard¹², by suspense 20 g of resin in 1 L of DW, then the mixture was left to settle down and washed several times with DW until getting a clear appearance. The suspension was filtered throughout Whatman No. 1 using the Buchner funnel under discharging. The resin was re-suspended in 0.25 M sodium chloride and sodium hydroxide solution. As mentioned above, the suspension was filtered again and washed several times with 0.25 M hydrochloric acid solution, followed by distilled water before it was equilibrated with 0.05 M phosphate buffer pH 7. The enzyme solution obtained from the previous step was applied to the DEAE-

cellulose column (2x25cm) equilibrated previously with 0.05M phosphate buffer pH7. Then, the column was washed with an equal volume of the same buffer while the attached proteins were stepwise eluted with gradual concentrations of sodium chloride (0.1–1 M). The flow rate throughout the column was 30ml/hrs, and the absorbance of each fraction was measured at 280 nm using a UV-VIS spectrophotometer. Enzyme activity was determined in each fraction.

Purification of L-asparaginase by gel filtration chromatography

Sephadex G-150 was prepared as recommended by Pharmacia Fine Chemicals Company. A quantity of Sephadex G-150 was suspended in 0.05 M phosphate buffer pH7, subjected to heating at 90C for 5 hrs to ensure the swelling of the beads, degassed, and packed in a glass column (2×40 cm), then equilibrated with the same buffer. The concentrated sample obtained from the previous step was applied to the column. Elution was achieved at a 30 mL/hr flow rate, and the same buffer was used for equilibration. The absorbance of each fraction was measured at 280 nm. Enzyme activity was also determined in each fraction 13.

Cytotoxic assay

Ahmed Murtada Jabria's 2013 breast cancer cell line has been established by an Iraqi breast cancer patient. The cell line was established from the primary tumor of a 70-year-old Iraqi woman with a histological diagnosis of infiltrating ductal carcinoma¹⁴. Cell lines were maintained in RPMI supplemented with 10% Fetal bovine, 100 units /mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA (Capricorn, German), reseeded at 50% confluence twice a week and incubated at 37 °C¹⁵. To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates¹⁶. Cell lines were seeded at 1×10^4 cells/well. After 24 hrs. or a confluent monolayer was achieved, cells were treated with the tested compound. Cell viability was measured after 72h of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT (Bio-World, UAS), and incubating the cells for 1.5 h at 37 °C. After removing the MTT solution, the remaining crystals in the wells were solubilized by adding 130 µL of DMSO (Santacruz Biotechnology, USA), followed by 37 °C incubation for 15 min with shaking⁵. The absorbance was determined on a microplate reader at 492 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated by the following equation¹⁷:

$$\% \text{ Cell viability} = (\text{Absorbance of treated cell} / \text{Absorbance of non-treated cell}) \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \text{cell viability}$$

Statistical analysis

The obtained data were statically analyzed using an unpaired t-test with GraphPad Prism 6. The values were presented as the mean ± SD of triplicate measurements.

RESULTS

Phenotypic Identification of LABs

Morphological assessments showed that all seven LAB isolates were lactobacilli. They could grow at 37 °C on MRS agar medium under micro-aerophilic conditions:

Purification of L-asparaginase

The crude culture had a total of 56.2 U with a protein content of 0.48. The specific activity was 1.56 U/mg protein, showing a purification fold of 1 mg protein content of The ammonium Sulphate. The concentrated enzyme preparation had a total of 30 U/mg protein, protein content of 0.21 mg/ ml, and the specific activity was 9.52 U/mg protein, showing a purification fold of 6.1 with the fractions collected after ammonium sulfate precipitation was loaded on the column-packed with DEAE cellulose (2x 25cm) column and fractions of 3 ml were collected. The precipitated enzyme was then purified by ion exchange chromatography (IEC) through a HiTrap DEAE-Sepharose Fast Flow column that was eluted in 50 mM Tris-HCl and 1 M NaCl, pH 8.5 at a flow rate of 1.0 ml/min.

The total activity was 29.4, with a protein content of 0.1mg. The specific activity of the purified enzyme was 14 U/mg of protein, showing a purification fold of 8.9 mg. Fractions containing high enzyme activity were then applied to the Sephadex G-150 gel filtration column. After ion exchange chromatography, The total activity was 28.8protein content of 0.04 mg/ ml, and the specific activity was 40 U/mg protein, showing a purification fold of 25.6 with the fractions collected after Gel filtration chromatography for L-. asparaginase

Cytotoxicity of L. asparaginase to cell line

To test the antiproliferative effects of L. asparaginase (3.125-100µg), a breast cancer cell line (AMJ) was treated at various doses. A Sigma Plot was used to determine the dose-response curve and IC50 values. The results showed a variable effect of treatments on cell lines (AMJ) proliferation. The exposure of the cell line to the enzyme showed a slight effect on the viability of the normal cell line (figure 1). The inhibition rates (IR%) are 30%,40%,50%, 60%,70.7%, and 80% of cell death occurred when 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml of drug used respectively and IC50 was 4.305 µg/ml.

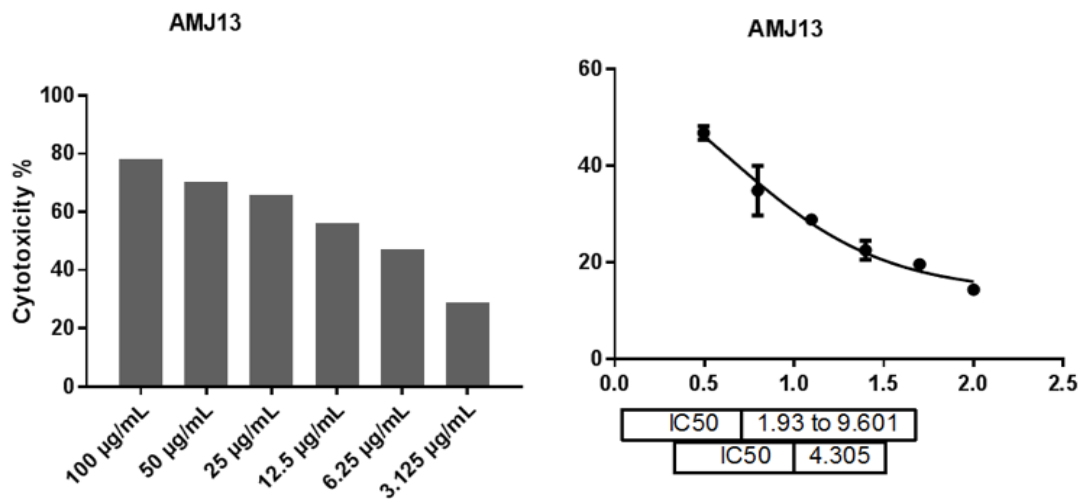


Figure 1. Cytotoxic effects of *L. asparaginase*

DISCUSSION

The results obtained from Gram staining and catalase activity and Api 50 results revealed that LAB isolates were Gram-positive and catalase-negative, and they are *Lactobacillus reuteri*.

Targeting amino acid metabolism has been safely and effectively employed for tumor therapy¹⁹. Tumor cells require huge amounts of asparagine to keep up with their rapid malignant growth. Therefore, L-asparagine is an essential amino acid for the growth of tumor cells. In contrast, the growth of normal cells is not dependent on its requirement as it can be synthesized in amounts sufficient for their metabolic needs with their enzyme L-asparagine synthetase (ASNS)^{20,21}. The presence of therapeutic asparaginase deprives tumor cells of an important growth factor by hydrolyzing L-asparagine into L-aspartic acid and ammonia. Afterward, tumor cells fail to survive because of their reduced ASNS levels²².

CONCLUSIONS

Breast cancer cell proliferation was significantly reduced by the cytotoxic action of isolated L-asparaginase from *Lactobacillus reuteri*, indicating its potential for further pharmaceutical application as an anticancer candidate.

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Conflicts of Interest: The authors declare no conflict of interest.

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