Original Article

A polyethylene glycol-based method for extraction of extracellular vesicles from *Lactobacillus casei* as vaccine delivery vehicle

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ABSTRACT

Introduction The secretion of extracellular vesicles (EVs) has been neglected in Gram-positive bacteria due to the absence of an outer membrane and the difficulties of proper visualization. Here we aimed to prove that *lactobacillus casei* can secrete extracellular vesicles. **Methods**: EVs were extracted from *Lactobacillus casei*, cultured in De Man, Rogosa and Sharpe broth, using a polyethylene glycol (PEG) solution. The characteristics of the EVs were analyzed by electron microscopy, Dynamic Light Scattering (DLS) and SDS-PAGE. **Results:** The electron microscopy showed rounded vesicles with average diameter of 300 nm. The protein content of this nanostructure was 2.5 mg/ml with a protein pattern within the range of 10-200 kDa. DLS result showed populations of approximately 300 nm while the extracted EVs had a negative zeta potential. **Conclusion:** A new method of producing functional molecules from probiotic bacteria was presented. Our results indicated EVs purity with acceptable conformation. Further investigations are necessary to elucidate the efficacy, practicality and mechanism of action of such EVs in clinical practices, especially for development of bio-compounds and vaccine delivery vehicles.

KEYWORDS: *Lactobacillus casei*, extracellular vesicles; polyethylene glycol.

INTRODUCTION

Probiotics possess health promoting properties. One of the most important probiotic strains belongs to Lactobacillus genus. Among Lactobacillus species, Lactobacillus casei is one of the most common probiotics with industrial applications. It has been reported to have positive effects on human health such as causing remarkable improvements in immunity [1, 2], allergies [3] and cholesterol levels [3]. Over the past few years, scientists have proven that probiotics and their bio-compound can have many benefits. For instance, they can inhibit human immunodeficiency virus (HIV) and herpes simplex virus (HSV) infections [4]. Moreover, components from Lactobacillus spp. can down-regulate the proinflammatory signaling pathways [5]. Also, exopolysaccharides of Lactobacilli have anti-elastase and anti-collagenase potentials on human fibroblast [6].
Interestingly, the probiotic lactobacilli used in the food industry could the best option to treat many infectious diseases. In fact, one of the properties of lactobacilli is their competition with the pathogens for the cell attachments [7]; as it has been confirmed that lactobacilli have anti-adhesive effects on certain pathogenic bacteria[8].
Lactic acid bacteria, are widely used in the food industry; therefore, the recognition of molecules derived from lactic acid bacteria can be useful to develop a new generation of functional compounds. One of the molecules secreted by bacteria is extracellular vesicles (EV)[9] which contain lipid bilayers that within 20–500 nm range in diameter. These structures do usually contain varied cargos, such as lipoproteins, nucleic acids, toxins and communication signals which are of great importance for the microbial pathogenesis and physiology [10]. Moreover, we have demonstrated that EVs can also be used as a delivery system [11]. It has been shown that E. coli EVs could be used as a vaccine vehicle system; however, due to presence of lipopolysaccharides in these nanostructures, a number of structural changes are needed [12].
Despite their great significance, the processes of EV isolation and characterization are still quite challenging and it would be essential to characterize an optimal method for this purpose. EV

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purification has been accomplished by at least four different methods, namely immunoaffinity [13], chromatography [14], ultracentrifugation and polymer-based precipitation [15]. Among these methods, ultracentrifugation is the most common one. The major advantage of ultracentrifugation is that it can manage large sample volumes for an improved throughput. Nevertheless, EV isolation by this method is time-consuming and a low yield of a degraded product is generally obtained. Various commercially prepared isolation kits have been recently offered; however their applications are limited due to their high cost and relatively low purity of the preparations [16].

The isolation of probiotic component is an important research priority and challenge for the food as well as the vaccine industries and other science sectors. In absence of a standard isolation technique for *L. casei* which has the benefits of lacking lipopolysaccharides in its structure and also being regarded as a safe probiotic bacterium, here we investigated a method for its EV purification using polyethylene glycol (PEG). Using this technique for the first time, we were able to isolate EVs from large volumes of media, rapidly and cost-effectively via low-speed centrifugations.

MATERIALS and METHODS

Bacterial strain and culture method

L. casei (ATCC 393 strains) was obtained from Iranian Biological Resource Center (Tehran, Iran) and was cultured in De Man, Rogosa and Sharpe agar (MRS; Sigma-Aldrich; Germany). In order to obtain more biomasses, a large volume of the bacterial culture was prepared (37°C, 24 h) in MRS broth [17].

Preparation of PEG solution for isolation of EVs

PEG solution is usually applied to concentrate virus particles. A virus enrichment method was modified to concentrate and isolate EVs of similar size and biochemical features. PEG (Sigma, 81260, Germany) with average Mw of 6000 Da was mixed with sodium chloride (1 M) and filtered water (ELGA Purelab Flex Purification System; 18.3 MegaOhm System, Germany). Afterwards, the stock solution was added to the bacterial culture supernatant of the same volume. Concentrations at 16% of PEG solution were evaluated in terms of ability to recover 20-1000 nm particles. In precipitation of the solutions, the concentration of sodium chloride was kept at 1 M [16].

Extraction of the EVs

A large *L. casei* bacterial culture (37°C) in MRS broth was prepared overnight in order to obtain more biomasses. From the bacterial culture, the vesicle-containing medium was centrifuged for 45 min at 6000 × g. Centrifugation was then performed again at 10000 × g for 30 min (4°C) to remove the cellular debris. After centrifugation, the medium was added at 4°C to an equal volume of 16% PEG solution. The sample was then completely mixed via inversion and was incubated overnight for a minimum of 12 h at 4°C. The samples were centrifuged the following day, using a tabletop centrifuge device at 3200 ×g (Eppendorf; model 5810 R with an S-4-104 swing bucket rotor; 3,214 g; Germany) for 1 h at 4°C. After the conical tubes were decanted, they were allowed to drain for 5 min, with occasional tapping to discard excess PEG [16]. Finally, particle-free sucrose was used to resuspend the samples. For further analyses, the sample subsets were stored at −80°C.

SDS-PAGE

Samples were loaded on 12% gel to determine the protein patterns following electrophoresis. The protein concentrations were measured using Nanodrop and Bradford assays, based on spectrophotometric methods.

Scanning Electron Microscopy (SEM) analysis

SEM was performed to determine the morphology and size of the EVs. The samples were placed in aluminum holders and then sputter coated using 5 nm of gold. The prepared samples were examined by SEM (KYKY Technology, China) [18].

Evaluation of EVs size via dynamic light scattering (DLS) and Zeta potential measurements

The size distribution and diameter of EVs which were resuspended in sucrose solution, were determined using DLS with a particle size analyzer (90Plus/BI-MAS Multi-Angle Analyzer, Brookhaven Instruments Corp, UK) [19]. A zeta potential analyzer (ZetaPlus; Brookhaven Instruments Corp, UK) was used to determine the zeta potential of EVs in 3% sucrose solution [19].

RESULTS

Evaluation of EVs by SDS-PAGE

Total protein concentrations of the EVs were analyzed by NanoDrop and Bradford assays. The evaluated concentrations of the purified total protein were 2.5 mg/ml and 2.31 mg/ml by Bradford and Nanodrop methods, respectively. The protein profile of the isolated EVs was in the range of 10-200 kDa as observed by SDS-PAGE method (Fig.1).

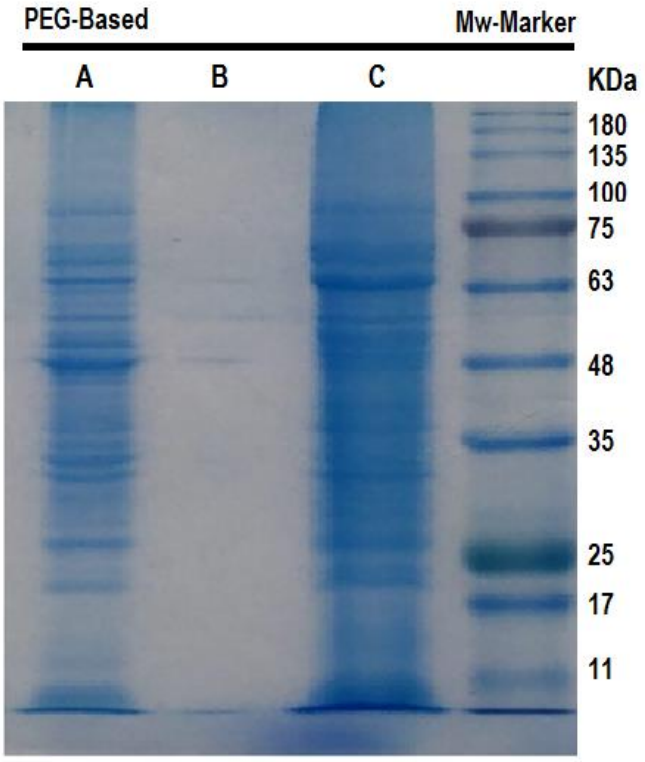

Fig.1. SDS-PAGE of EVs purified from *L. casei*, stained by Coomassie method. SDS-PAGE of *L. casei* derived EVs by ultracentrifugation (C lane) and PEG-based method (A lane) and B lane: empty. The ladder shows the protein bands between 11-180 kDa.

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The electron microscopy showed spherical vesicles with specific physicochemical properties with diameters ranged between 200–400 nm (Fig. 2).

Physical characterization of the L. casei vesicles

EVs preparations from L. casei cultures were evaluated by DLS technique to determine the size distribution of the EVs. The DLS analysis showed 2 populations of approximately 50-500 nm in preparations from the PEG-based method (Fig. 3).

Activity decreased significantly by 45.85% after EDC-NS3 immunization but CDC-NS3 immunization caused a non-significant decrease (3.14%) in its activity.

Zeta Potential Measurements

Zeta potential of the isolated EVs was determined for two purification techniques. Based on our data, the measurements of the zeta potential of the vesicles were negative (−11 mV), as shown in Fig. 4.

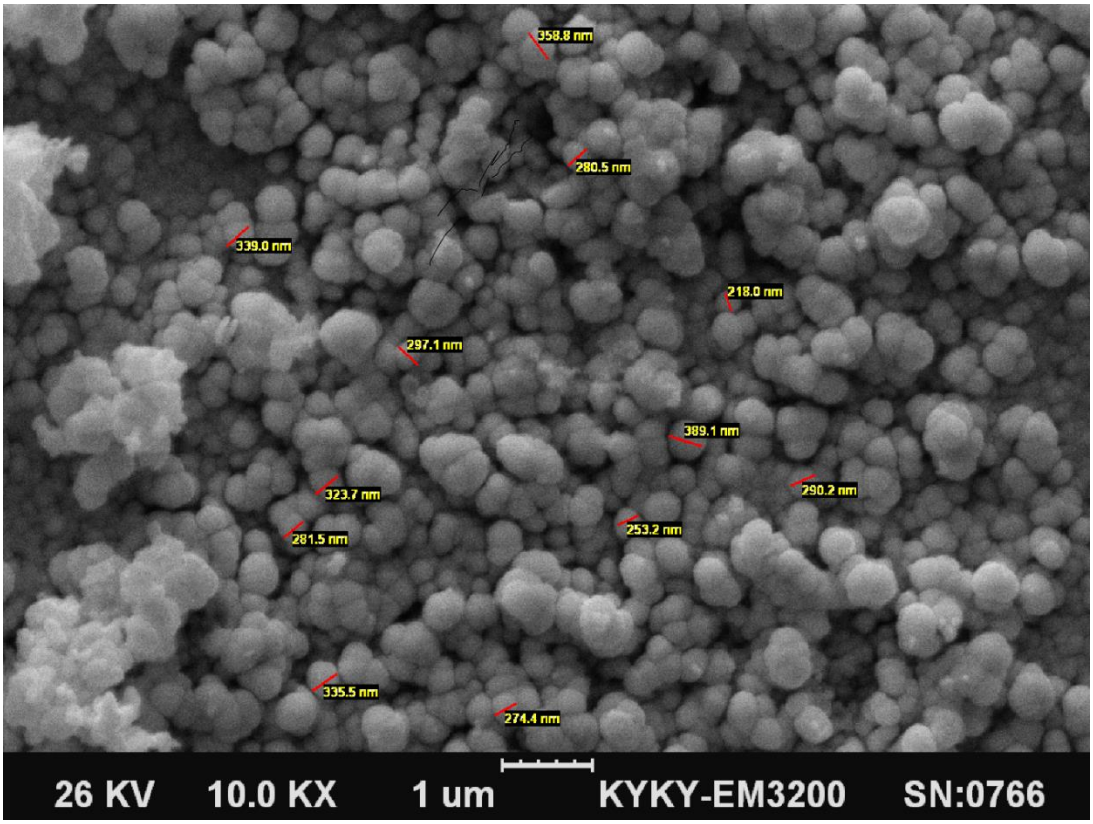

Fig.2. SEM micrographs of the EVs preparations by PEG solution (magnification: ×10K).

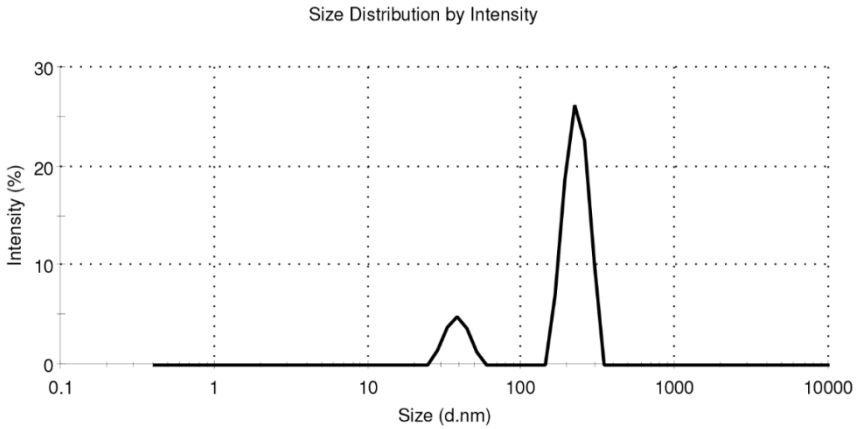

Fig. 3. Size distribution report by intensity of the EVs from the PEG-based solution.

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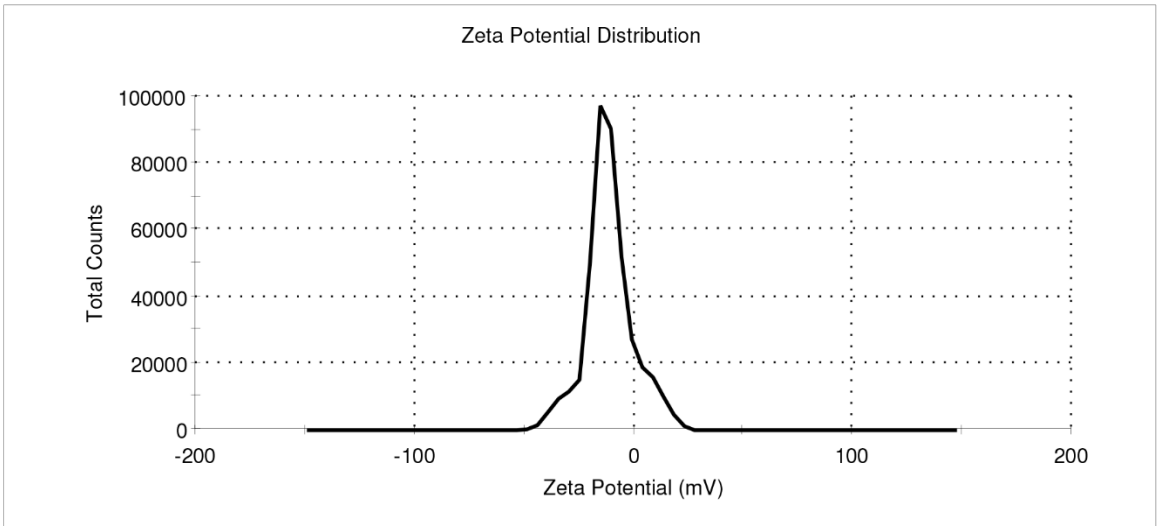

Fig. 4. Zeta potential report of the EVs extracted by the PEG-based solution.

DISCUSSION

Due to the importance of probiotics in basic scientific studies and commercial applications, we attempted to study a new method of EVs from a member of the probiotic bacteria using a PEG-based solution. Besides increasing food productions based on probiotics, a large number of publications have shown that probiotic bacteria have significant positive effects on human health. For example, it has been shown that components from Lactobacillus spp. can down-regulate the proinflammatory signaling pathways [5]. Moreover, derivatives from probiotics like EVs have opened the door for development of a new generation of functional compounds, such as vaccine vehicles. In this study, we demonstrated that L. casei which is one of the most important probiotics, is able to produce and release EVs. Many methods have been developed for isolation and characterization of EVs in recent years and it could be assumed that any of these methods could have direct effects on their downstream analyses. Among many different techniques to identify EVs [20-22], here we used five of them.

Observations by electron microscopy are among the most common techniques for detecting extracellular vesicles in recent years [23]. For instance, the structure and size of *Staphylococcus aureus* derived EVs have been studied using Transmission Electron Microscopy (TEM) [24]. Moreover, Li et al. (2017) have isolated EVs from *Lactobacillus plantarum* and have shown by electron microscopy that the size of these nanostructures is 30-300 nm. According to many studies, the diameter of EVs from Gram-positive organisms are ~20–200 nm, which is similar to the size of EVs from Gram-negative bacteria [10]. It is noteworthy that the size of EVs from Gram-positive bacteria could be varied. This feature indicates that the synthesis of EVs is regulated in a variety of ways, although vesiculogenesis is considered a general procedure. We performed SEM in this study to determine the spatial structure and size of these nanostructures which had an average diameter between 200–400 nm.

Our evaluations of total protein concentrations and profile of *L. casei* derived-EV were based on similar methods used by other researchers [23, 25]**.** Our data indicated that the concentration of the EVs by the PEG-based method was approximately 2.5 mg/ml. Moreover, the Mw of *L. casei* derived-EVs ranged from 10 to 200 kDa, confirmed by SDS-PAGE.

The physical characterizations of *L. casei* vesicles were done by DLS analysis and zeta potential measurements. DLS analysis by Brown et al. has revealed two populations of approximately 50 and 150–250 nm in EVs preparations from Bacillus subtilis strain 168 [26]. After isolating EVs from *L. casei* by the PEG-based method, we noted that the isolated vesicles produced different DLS profiles. Our DLS analysis showed heterogeneous diameters, in consistence with the sizes predicted by the electron microscopic studies; nonetheless, this technique overestimates or underrates the diameter of the vesicles. The dimensions reported in DLS analysis were consistent with the SEM measurements.

EVs extracted by the PEG-based method were negatively charged according to zeta potential analyses and had a high zeta potential. The small zeta potentials of the EVs show their instability in solution and indicate the need for caution in storing and handling EVs at temperatures other than −80°C. These results were similar to the extracellular vesicles described by Dean et al.[27]. When the potential is from 0 to ±5, we call it small, and in this situation, attractive forces greater than repulsion and dispersion are disrupted and flocculation happens. Therefore, EVs with low zeta potential (negative or positive) are electrically unstable and tend to coagulate. Therefore, the extraction methods can affect the zeta potential and the stability of the EVs.

Ultracentrifugation is still the standard method for EV isolation, however, it requires parameters which are difficult to optimize, including the g-force, the rotor type (fixed angle or swinging bucket), and centrifugal radius. Moreover, it is relatively time-consuming and sensitive and requires access to an ultracentrifuge apparatus and prior trainings. The EVs integrity may also be impaired following prolonged ultracentrifugation at high speeds [28] and some observations have suggested that the ultracentrifuge method produces vesicles with reduced biological activities [29]. On the other hand, the use of commercial kits is not cost-effective.

In conclusion, our results proved that EVs purity and conformation were within an acceptable range and by this method EVs can be easily and inexpensively isolated from

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probiotic bacteria using a low-speed centrifugation. In addition, the possibility to recover many vesicles with higher resistance makes this method advantageous. Since EVs can deliver bacterial products to the host, extensive efforts are being made to deliver immunogenic antigens by these structures. The positive effects of vaccination with EVs of Gram-positive bacteria have been carried out against *Clostridium perfringens*, *S. pneumoniae*, *B. anthracis*, *M. tuberculosis*, and *S. aureus*. Based on animal models, the above mentioned EVs vaccinations have prolonged the survival time upon lethal challenges [30-33]. The identification of active components of *L.casei* secreted EVs and its potential therapeutic use remain to be investigated. Further researches are necessary to elucidate the efficacy, the practicality and the mechanisms of action of such EVs in clinical practices, especially as vaccine delivery vehicles.

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CONFLICT OF INTEREST

None of the authors had any personal or financial conflict of interest.

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