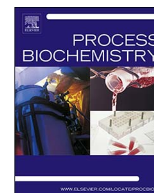
ARTICLE IN PRESS

Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Bacteriocin partitioning from a clarified fermentation broth of *Lactobacillus plantarum* ST16Pa in aqueous two-phase systems with sodium sulfate and choline-based salts as additives

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A R T I C L E I N F O

Keywords:
Bacteriocin partitioning
Aqueous two-phase system
Poly(ethylene glycol) (PEG)
Sodium polyacrylate (NaPA)
Choline chloride ([Ch]Cl)
Lactic acid bacteria (LAB)

A B S T R A C T

The partitioning of bacteriocin, a promising alternative to chemical preservatives, in a novel inexpensive and stable aqueous two-phase system (ATPS) comprising poly(ethylene glycol) (PEG) and sodium polyacrylate (NaPA) was studied. The ATPS was generated by mixing both polymers with Na_2SO_4 or choline chloride ([Ch]Cl) and a bacteriocin extract from the fermented broth of *Lactobacillus plantarum* ST16Pa. Bacteriocin showed stability at different pH values (3.0–8.0) and temperatures (50–80 °C), as well as in the presence of ATPS components. Hydrophobic and electrostatic interactions were found to be the major driving forces for bacteriocin partitioning. The peptide partitioned preferentially to the PEG-rich phase (partition coefficient, $K_{Bact} > 1$). However, the highest partition coefficient was achieved in the polymeric-based ATPS using [Ch]Cl as additive, as follows: 8 wt% PEG 10,000 g/mol/8 wt% NaPA 8,000 g/mol/0.5 M [Ch]Cl, resulting in a K_{Bact} equal to 32. Moreover, these conditions promoted high selectivity ($S = 62.7$), since the greater part of total proteins partitioned into the NaPA-rich phase ($K_{prot} = 0.51$). In conclusion, ATPS composed of PEG/NaPA using choline-based salts as additive can be considered mainly as a first step for bacteriocin extraction from fermented broth by applying low polymer content and mild conditions.

1. Introduction

The wide use of chemical preservatives, such as nitrites and sulfur dioxide, to extend the shelf life of foods may cause adverse effects on human health and on the nutritive value of food [1]. For this reason, consumers are increasingly worried about the amount of chemical additives present in their diet, which has led to a growing demand for natural or chemical-free food [2]. This demand, coupled with the increasing desire for minimally processed food with long shelf life, has attracted research interest in finding natural preservatives [3]. In this sense, bacteriocins, mostly produced by lactic acid bacteria (LAB), are a promising alternative to the preservatives available in the market [4].

Bacteriocins are defined as bacterially produced, heat-stable peptides that are active against bacteria except its producer [5]. Several purification protocols have been developed to extract bacteriocin from LAB cultures [6]. Generally, the first step involves the concentration of bacteriocin from the culture supernatant using ammonium sulfate precipitation [7]. This method does not provide a high degree of purity; therefore, further steps using preparative isoelectric focusing and/or multiple chromatographic techniques, including cation exchange, gel filtration, hydrophobic interaction, and reverse-phase liquid chromatography, are necessary to achieve highly pure bacteriocins [8]. However, these methods have their own drawbacks, such as high operation costs and protocols that include several steps, resulting in low yield [9].

In the search for alternative methods, liquid–liquid extractions conducted in various types of aqueous two-phase complex-fluid systems have been investigated for bioseparation [10]. An aqueous two-phase system (ATPS) can be applied for bacteriocins extraction directly from the fermented medium, leading to a simplification of the overall purification protocol [11]. Some studies have demonstrated that an ATPS

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https://doi.org/10.1016/j.procbio.2017.11.018
Received 12 July 2017; Received in revised form 23 November 2017; Accepted 24 November 2017
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Please cite this article as: Silva Sabo, S.d., Process Biochemistry (2017), https://doi.org/10.1016/j.procbio.2017.11.018

can be formed from polyethylene glycol (PEG) and sodium polyacrylate (NaPA), resulting in a PEG-enriched top-phase and a NaPA-enriched bottom phase [12–19]. PEG/NaPA systems have some advantages, such as low viscosity, ease of handling, rapid phase separation, relatively low-cost chemicals, and are environmentally friendly, compared with traditional polymer-polymer systems (PEG/dextran) [13,14]. PEG is an uncharged polymer, while NaPA is negatively charged, thus the entropic penalty of counter-ion compartmentalization leads to a minimal concentration of salt being required to form the two aqueous phases [14].

In previous studies, PEG/NaPA based ATPSs have been used to partition different biomolecules, such as green fluorescent protein [15], hemoglobin, lysozyme, glucose-6-phosphate dehydrogenase [16], myoglobin, ovalbumin [17], cytochrome c [18], proteases [13], and amyloglucosidase [19]. Remarkably, the use of a third component, *i.e.* salts, has been proposed to improve ATPS potential. The addition of salts may result in changes to the polymer-polymer interactions because of electrostatic screening. Although salts distribute almost evenly between the two phases of an ATPS, there is a small, but significant, difference in the partition coefficient of electrolytes, which may generate an electrostatic potential difference between the phases, thereby improving the partitioning of charged molecules [20–22]. Parallelly, the effect of electrolytes might also induce changes in the solution properties, *i.e.* causing salting in and salting out effects [23–25].

Although a few studies using bacteriocin partitioning/recovery using PEG/salt-based ATPSs [26–30], and with an aqueous two-phase micellar system (ATPMS) [31,32], have been evaluated, PEG/NaPA systems composed of inorganic salts or choline-based salts have not been proposed before. In this context, and based on the advantages mentioned above, the present study investigated a PEG/NaPA based ATPS with Na_2SO_4 or choline chloride ([Ch]Cl) as additives to extract bacteriocin from the fermented broth of *Lactobacillus plantarum* ST16Pa. Preliminary experiments were performed to evaluate the stability of bacteriocin in solutions of ATPS components (PEG, NaPA, and salts). Subsequently, a multifactorial experimental design was used to estimate the effects of pH and temperature on bacteriocin partitioning in ATPS. To the best of our knowledge, this is the first study on the partitioning of bacteriocin produced by *L. plantarum* ST16Pa in a polymer-polymer system.

2. Material and methods

2.1. Materials

PEG with molar masses of 2,000, 6,000, and 10,000 g/mol, were purchased from Merck (Hohenbrunn, Germany). Polyacrylic acid (NaPA) 8,000 g/mol (45 wt%) and the salts, sodium sulfate (Na_2SO_4) and choline chloride ([Ch]Cl), were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solutions were prepared in sodium acetate 0.1 M buffer, pH 5.0, with water purified using a Millipore Milli-Q system (Bedford, MA, USA). The glassware used was washed in a 50:50 ethanol:1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water and, finally, dried in an oven at 70 °C for 1 h. All other reagents were of analytical grade and used as received.

2.2. Microbial cultures and fermentation conditions

The microorganism used was the *L. plantarum* strain ST16Pa, isolated by Todorov et al. [33] from a papaya species. As a microorganism indicative of bacteriocin antimicrobial activity, it was used the *Listeria innocua* strain 6a CLIST 2860 (AL224/07), isolated from a dry-fermented sausage sample and provided by Collection of *Listeria* (CLIST) from Fundação Oswaldo Cruz (FioCruz, Rio de Janeiro, Brazil).

For bacteriocin production by *L. plantarum* ST16Pa, first, the strain was reactivated by adding 1 mL of cryopreserved strain stock into a 250 mL Erlenmeyer flask containing 100 mL of Man, Rogosa and Sharpe (MRS) broth (DIFCO, Detroit, MI, USA) and incubating at 30 °C on a rotatory shaker at 100 rpm for 24 h. Subsequently, 10 vv% of this culture was used to inoculate 1 L Erlenmeyer flasks containing 500 mL of MRS broth, also incubated at 30 °C for 24 h.

To grow the bioindicator strain, *L. innocua* 6a CLIST 2860, 10 mL of the Brain Heart Infusion (BHI) broth (DIFCO, Detroit, MI, USA) was inoculated with 1 mL of cryopreserved strain stock and incubated overnight at 37 °C on a rotatory shaker at 100 rpm.

2.3. Determination of bacteriocin antimicrobial activity

The 24 h culture of *L. plantarum* ST16Pa was centrifuged at 25,750 × *g* for 15 min. The pH of the resulting cell-free supernatant (CFS) was adjusted to 6.0–6.5 with 1 M NaOH to eliminate organic acids, and heated to 80 °C for 10 min to inactivate proteases. Finally, the CFS was filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA), and later tested against the bioindicator *L. innocua* strain 6a CLIST 2860. The test was performed by the agar diffusion method, in which the bioindicator culture broth was diluted 100-fold approximately 10^7 Colony Forming Units per mL (CFU/mL). One milliliter of this dilution was transferred to a Petri dish (90 × 15 mm) containing 10 mL of melted BHI soft agar (containing 0.75 wv% agar). Then, 20 μL of the CFS was spotted onto the agar surface. A period of 3 h was allowed for the diffusion of the supernatant at 25 °C and then the plates were incubated at 37 °C for 24 h. Subsequently, the inhibition halos were measured in four directions using a digital caliper gauge (Lee Tools, model 684132) and the antimicrobial activity of bacteriocin (A_{Bact}), defined in arbitrary units (AU), was calculated based on the method of Sidek et al. [30]. The equation was as follows:

$$A_{\mathrm{Bact}} = \left(\frac{\pi R^2}{\mathrm{v}}\right) = \mathrm{AU/mL} \tag{1}$$

where πR^2 is the inhibition (clear) zone area of the halo (in cm^2) and V is the volume (in mL) of the CFS (sample) dropped onto the agar surface. Some results were presented as $\%A_{Bact}$, considering the initial bacteriocin activity (around 68.35 AU/mL) as 100%. All experiments were performed in triplicate and average values were presented.

2.4. Determination of the total protein concentration

The total protein concentration was determined using the bicinchoninic acid method (BCA), which is compatible with the polymers used. Samples containing proteins (100 μL) and 2 mL of the BCA working reagent, prepared according to the manufacturer's instructions, were added to a test tube. After 30 min, the optical density at 562 nm was determined in a spectrophotometer, using deionized water as a blank. Absorbance values were correlated with protein concentration based on a calibration curve using bovine serum albumin (BSA) solutions from 0 to 1,000 μg/mL (equation obtained: y (ABS) = 0.0011 x (μg/mL) + 0.0245, R^2 = 0.99).

2.5. Bacteriocin activity in different conditions

The effect of pH and temperature on bacteriocin activity after 1 h was studied using a 2^2 central composite design. Bacteriocin activity was defined as the residual antimicrobial activity after exposure to the pH and temperatures investigated; these values (AU/mL) were correlated in terms of bacteriocin stability. A set of 12 experiments, which contained a factorial or fractional factorial matrix, with center points and star points to allow the estimation of the curvature, was performed. The range and levels of the components under study are given in Table 1. The pH of the solutions was adjusted using 5 M HCl or 5 M NaOH. Subsequently, we evaluated the influence of the ATPS components, namely PEG 10,000 g/mol, NaPA 8,000 g/mol, Na_2SO_4, and

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