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Liquid-liquid extraction in the presence of electrolytes of nisin and green fluorescent protein (GFPuv)

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Abstract

In the biotechnology field, it has been suggested that extractions in two-phase aqueous complex-fluid systems can possibly be used instead of, or as complementary processes to, the more typical chromatographic operations, to reduce the cost of the downstream processing of many biological products (Lam et al., 2004; Mazzola et al., 2006). This method offer attractive conditions to be applied in this study, thereby two-phase systems can be exploited in separation science for the extraction/purification of desired biomolecules directly on the culture medium (Mazzola et al., 2008). This study aimed to evaluate the aqueous two phase system (ATPS) composed by a nonionic surfactant, Triton X-114 (TX), in presence or absence of electrolytes, to separate two interesting biomolecules: nisin and recombinant green fluorescent protein (GFP). Results indicated that nisin partitions preferentially to the micelle rich-phase, with significant antimicrobial activity increase (up to 10-fold). GFP partitioned evenly between the phases in TX system without electrolytes.

1 Introduction

The industrial production of proteins and other biomolecules depend significantly on the extraction/purification processes. The development of techniques and methods for separation and purification of proteins has been essential for many advances in biotechnology (Wilcheck et al., 1999). In this sense, one of the techniques that can be considered promising is liquid-liquid extraction. Various liquid-liquid extraction methods that use different types of two-phase aqueous complex-fluid systems have been proposed to address a wide variety of separation needs in the chemical, biotechnological, and environmental fields. In general, these methods offer the possible advantages of versatility, scalability, cost effectiveness, and environmental friendliness compared to the conventional methods that they may replace. In the biotechnology field, it has been suggested that extractions in two-phase aqueous complex-fluid systems can possibly be used instead of, or as complementary processes to, the more typical chromatographic operations, to reduce the cost of the downstream processing of many biological products (Lam et al., 2004; Mazzola et al., 2006).

In particular, two-phase aqueous micellar systems, formed by some surfactants at certain conditions, have been proposed as an attractive option, to be used in bioseparations (Liu et al., 1998). In these systems an aqueous surfactant solution, under the appropriate solution conditions, spontaneously separates into two predominantly aqueous, yet immiscible, liquid phases, one of which has a greater concentration of micelles than the other (Liu et al., 1998).

The difference between the physicochemical environments in the micelle-rich phase and in the micelle-poor phase forms the basis of an effective separation and makes two-phase aqueous micellar systems a convenient and potentially useful method for the separation, purification, and concentration of biomaterials (Liu et al., 1996).

This method offer attractive conditions to be applied in this study, thereby two-phase systems can be exploited in separation science for the extraction/purification of desired biomolecules directly on the culture medium (Mazzola et al., 2008).

Nisin is an extracellular antimicrobial peptide, discovered in 1928, its utilization are approved by Food and Drugs Administration (FDA) (Hansen, 1994; Vessoni Penna and Moraes, 2002), meeting the requirements of safe food with fewer chemical additives (Chol et al., 2000). This peptide has large antimicrobial activity spectrum against Gram-positive bacteria and their spores (Delves-Broughton et al., 1996). Other applications of nisin include dental care products (Turner et al., 2004), pharmaceutical products such as stomach ulcers and colon infection treatment and potential birth control (Aranha et al., 2004; Dubois, 1995). Recently the optimization of nisin production has been reported. Jozala and co-workers (2005, 2007) and Vessoni Penna and co-workers (2005, 2006) studied nisin expression using different media compounds based on skimmed milk diluted with synthetic media (MRS and M17) and with nutrients added. Diluted skimmed milk was observed to be the best culture media for nisin production and activity by *L. lactis*.

The GFP, expressed by *Escherichia coli* DH5-α, is widely applied with as biossensor and can be detected by spectrofluorometry or using hand UV lamp, it has became a versatile tool for a variety of biotechnological uses and as a potential biological indicator, for preservation of manufactured and processed products (Vessoni Penna et al., 2004). GFP is an excellent biosensor due to its ability to be easily monitored in a wide variety of applications. Enzymes and proteins have been used as biological indicators to evaluate the immediate efficacy of industrial procedures, such as blanching, pasteurization, and disinfection treatments, as well as to monitor the satisfactory preservation of a product subjected to disinfection or sterilization.

This study aimed to evaluate the aqueous two phase system (ATPS) composed by a nonionic surfactant, Triton X-114 (TX), in presence or absence of electrolytes, to separate two interesting biomolecules: nisin and recombinant green fluorescent protein (GFP).

2 Materials and Methods

The biomolecules were obtained from commercial and produced way, standard nisin was provide by Nisin^{TM} (Sigma, St. Louis, MO) and GFP expressed by transformed *E. coli*; produced nisin was skimmed milk fermented by *L. lactis* and GFP was complex media fermented by *E. coli* DH5-α. The system was composed by 2% w/w TX with and without $MgSO_4$ or $(NH4)_2SO_4$. The phase diagram of the TX in buffer was obtained by the cloud-point method. Buffered solutions of the 2%TX with or without electrolytes, each with a total volume of 3 mL, were prepared in graduated 10-mL test tubes. Each solution was placed in a thermo-regulated device set at a temperature of 36.2ºC for 2h to attain partitioning equilibrium. Nisin activity was determined by agar diffusion with *L. sakei* as bioindicator. GFP concentration was determined by fluorimetry.

The biomolecules were purchased and biosynthesized, standard nisin was provides by Sigma (St. Louis, MO) and GFP was expressed by modified *E. coli* and purified; produced nisin was skimmed milk fermented by *L. Lactis*. The initial concentration of nisin in each system was 3 log AU/mL, and the initial concentration of GFP was 18µg/mL.

The system was composed by 2% w/w TX with and without $MgSO_4$ or $(NH4)_2SO_4$. The phase diagram of the TX in buffer was obtained by the cloud-point method. Buffered solutions of the 2%TX with or without electrolytes, each with a total volume of 3 mL, were prepared in graduated 10-mL test tubes. Each solution was placed in a thermo-regulated device set at a

temperature of 36.2ºC for 2h to attain partitioning equilibrium. After 2h the two coexisting micellar phases formed were withdrawn separately and the biomolecules concentration was determined in each micellar phase. Nisin activity was determined by agar diffusion with *Lactobacillus sakei* as bioindicator. GFP concentration was determined by fluorimetry.

3 Results

Since it is often observed that the phase behavior of aqueous micellar solutions can be sensitive to the presence of electrolytes and impurities (Balzer and Lüders, 2000), it was first necessary to study how the TX/buffer phase diagram, that is, the temperature versus TX concentration coexistence curve, changes in the presence of these components. The phase diagram of the TX/buffer system in the presence of the different electrolytes was therefore mapped, and the results are presented in Figure 1. The experimentally measured coexistence curve, in the absence of any extra components, was and is also presented for comparison purposes.

As can be seen, the coexistence curves in the presence of the electrolytes have considerably shifted when compared to the TX/buffer curve in the absence of salts.

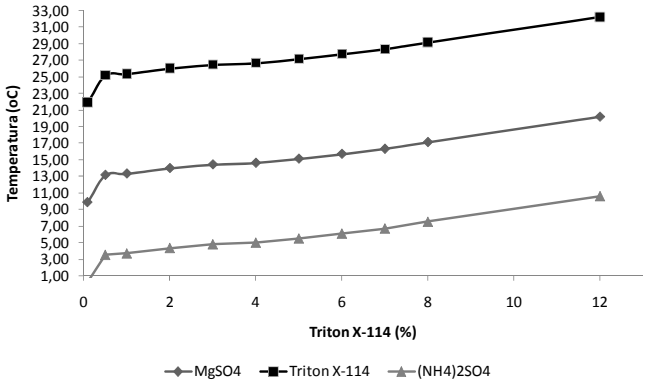

Figure 1. Phase diagram of Triton X-114/buffer system in the absence and in the presence of eletroctrolytes. The squares (■) represent the addition of $MgSO_4$; the losangles (♦) represents the coexistence curve for TX/buffer and the triangles (▲) represent the addition of $(NH_4)_2SO_4$.

The addition of salts in an ATPMS promotes a stronger electrostatic interaction between the water molecules compared to the hydrogen bonds between the head and the tail of the surfactant monomers. This phenomenon drives the water molecules to solvate the ions from the salts and the amphiphilic molecules interact more intensely, if compared to the absence of electrolytes (Carale, 1993).

The ions, such as sulphate, phosphate and chlorine, can increase the water structure and stabilize the structure of proteins, in this case they are called cosmotropic ions. On the other hand, there are some ions (perchlorite, thiocyanate, i.e.) which decrease the structure of the water and destabilizing the structure of proteins, being called chaotropic. The micelle formation is also changed by the presence of salts, once the critical micellar concentration (CMC) and the aggregation number (number of monomers in each micelle) are dependent on the solvation of ions (Anacker, Ghose, 1963; Tonova, Lazarova, 2005), also, the binding of the ions to the micelles affects the the organization and the electrostatic interaction between the polar heads and the tails of the surfactant monomers (Quina, Chaimovich, 1979; Brochsztain et al., 1990).

After the determination of the coexistence curves, systems containing nisin or GFP in the presence and in the absence of electrolytes were prepared and placed in a transparent thermo-regulated device whose temperature was controlled to within 0.02^{o}C.

In initial partitions, nisin was driven to the micelle-rich phase in the TX/buffer system, in the presence of the electrolytes similar behavior was observed, but the presence of $MgSO_4$ enhanced the most the partition of nisin to the micelle-rich phase (Figure 2). Results indicated that nisin partitions preferentially to the micelle rich-phase, with significant antimicrobial activity increase (up to 10-fold).

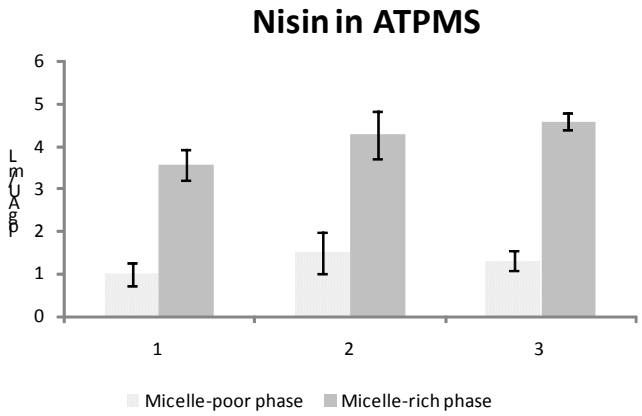

Figure 2. Nisin activity in the micelle-poor phase (gray and white bars) and in the micelle-rich phase (solid gay bars), after partitioning in ATPMS. (1) TX/buffer system; (2) TX/buffer and $(NH_4)_2SO_4$; (3) TX/buffer and $MgSO_4$. The error bars represent 95% confidence limits for the measurements.

The green fluorescent protein, in the TX/buffer system partitioned evenly between the phases, while in the presence of both salts GFP partitioned extremely to the micelle-poor phase (Figure 3).

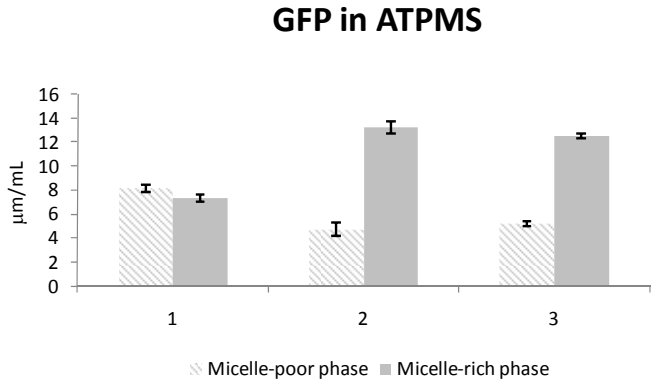

Figure 3. Green fluorescent proteins concentration in the micelle-poor phase (gray and white bars) and in the micelle-rich phase (solid gay bars), after partitioning in ATPMS. (1) TX/buffer system; (2) TX/buffer and $(NH_4)_2SO_4$; (3) TX/buffer and $MgSO_4$. The error bars represent 95% confidence limits for the measurements.

Different proteins are not soluble in water, but their solubility increases in the presence of electrolytes reaching a limit that can vary according to the protein and the chosen salt, this is called salting-in and can be explained by the interaction of the ions (positive and negative) in

the solution with the charged sites on the protein molecule, softening the interaction amongst them. For this reason, the electrostatic effect provided by diluted solutions containing salts is an important factor to the increase of solubility of proteins.

When the salt concentration is increased, some of the water molecules are attracted by the salt ions, decreasing the number of water molecules available to interact with the proteins. As a result of the increased demand for solvent molecules, the protein-protein interactions are stronger than the solvent-solute interactions; the protein molecules coagulate by forming hydrophobic interactions with each other. This process is known as salting out, and is caused by the presence of di or trivalent ions ($(NH_4)_2SO_4$, Na_2SO_4, i.e.)

The addition of salt, even milimolar concentrations, influences the partitioning of charged molecules. Although the ions partitions quite evenly between the phases, the small difference in their behavior in the phases creates a dielectrical potential that affects the biomolecules partition (SARUBBO et al., 2000). The addition of salts in ATPMS containing Triton X-114 influences the partitioning of both nisin and GFP, being a good strategy for extracting and purification of these biomolecules from the fermentation broth where they are biosynthesized.

The successful implementation of protein partitioning from a mixture containing the target biomolecule and other compounds, in a two-phase aqueous micellar system, presented in this work, represents an important step towards developing a novel separation method for proteins of interest.

4 Conclusions

The partitioning behavior of the biomolecules, nisin and GFP, in aqueous two-phase micellar systems in presence of electrolytes was investigated experimentally, showing that the target biomolecules can be extracted from respective media, either commercially available or produced by our research group. Increasing in activity of nisin and GFP concentration, after partitioning encourages further researches aiming to optimize the biomolecules extraction using simple tools as temperature variations, amongst others. In conclusion, the successful implementation of proteins partitioning, directly from a suspension containing the biomolecules and other unknown compounds, in a aqueous two-phase micellar system, presented in research, represents an important step towards developing a cost-effective separation method, and more generally, for other biomolecules of interest.

Acknowledgments

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References

Wilchek, M., Miron, T. (1999). Thirty years of affinity chromatography. *Reactive and. Functional Polymers*. 41, 263-268.

Lam, H., Kavoosi, M., Haynes, C.A., Wang, D.I.C., Blankschtein, D. (2004). Affinity-enhanced protein partitioning in decyl beta-d-glucopyranoside two-phase aqueous micellar systems. *Biotechnology and Bioengineering*, 89, 381–392.

Mazzola, P.G., H. Lam, H., Kavoosi, M., Haynes, C.A., Pessoa Júnior, A., Vessoni Penna, T.C., Blankschtein, D. (2006). Affinity-tagged green fluorescent protein (GFP) extraction from a clarified E. coli cell lysate using a two-phase aqueous micellar system., Biotechnology and Bioengineering, 93, 998–1004.

Liu, C.L., Kamei, D.T., King, J.A., Wang, D.I.C., Blankschtein, D. (1998). Separation of proteins and viruses using two-aqueous micellar systems. *Journal of Chromatography B*,

711, 127–138.
Liu, C.L., Nikas, Y.J., Blankschtein, D. (1996). Novel bioseparations using two-phase aqueous micellar systems, *Biotechnology and Bioengineering*, 52, 185–192.
Mazzola, P.G., Lopes, A.M., Hasmann, F.A., Jozala, A.F., Vessoni Penna, T.C., Magalhaes, P.O. Rangel-Yagui, C.O., Pessoa Júnior, A. (2008). Liquid-liquid extraction of biomolecules: an overview and update of the main techniques. *Journal of Chemical Technology and Biotechnology,* 83, 143-157.
Hansen, J.N. (1994). Nisin as a model of food preservative. *Critical Reviews in Food Science and Nutrition*, 69–93.
Vessoni Penna, T.C., Moraes, D.A. (2002). Optimization of nisin production by Lactococcus lactis. *Applied Biochemistry and Biotechnology*, 98-100, 775-789.
Chol, H.J., Cheigh, C.I., Kim, S.B., Pyun, Y.R. (2000). Production of a nisin-like bacteriocin by Lactococcus lactis subsp. lactis A164 isolated from Kimchi. *Journal of Applied Microbiology*, 88, 563-57.
Delves-Broughton, J., Blackburn, P., Evans, R.J., Hugenholtz, J. (1996). Applications of the bacteriocin, nisin. *Antonie Leeuwenhoek*, 69(2), 193-202.
Turner, S.R., Love, R.M., Lyons, K.M. (2004). An in-vitro investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine. *Internacional Endodontic Journal,* 37, 664–671.
Aranha, C., Gupta, S., Reddy, K.V.R. (2004). Contraceptive efficacy of antimicrobial peptide Nisin: in vitro and in vivo studies. *Contraception: An Internacional Journal*, 69, 333–338.
Dubois, A. (1995). Spiral bacteria in the human stomach: the gastric Helicobacter, Emerging *Infection Diseases Journal*, 1(3), 79–88.
Jozala, A.F, Novaes, L.C.L., Cholewa, O., Moraes, D., Vessoni Penna, T.C. (2005). Increased of Nisin Produced by Lactococcus lactis in Different Media. *African Journal of Biotechnology*, 4(3), 262-265.
Jozala, A.F, Andrade, M.S., Arauz, L.J., Pessoa Júnior, A., Vessoni Penna, T.C. (2007). Nisin production utilizing skimmed milk aiming to reduce process cost. *Applied Biochemistry and Biotechnology*, 136, 515-528.
Vessoni Penna, T.C., Jozala, A.F, Novaes, L.C.L., A. Pessoa Júnior, A., Cholewa, O. (2005). Production of nisin by Lactococcus lactis in media with skimmed milk. *Applied Biochemistry and Biotechnology*, 121–124, 1–20.
Vessoni Penna, T.C., Jozala, A.F, Gentille, T.R., Pessoa Júnior, A., Cholewa, O. (2006). Detection of nisin expression by Lactococcus lactis using two sensitive bacteria to associate the effects of nisin with EDTA, *Applied Biochemistry and Biotechnology* , 121–124, 334–346.
Carale M.T.R. *Salt effects on micellization, micellar growth, and phase behavior of aqueous solutions of nonionic surfactants*. Cambridge, 1993. 189p. Tese de Doutorado - Massachusetts Institute of Technology.
Anacker, E.W.; Ghose, H.M. (1963). Counterions and micelle size. 1. Light scattering by solutions of dodecyltrimethylammonium salts. *Journal of Physical Chemistry*, 67, 8, 1713-1716.
Tonova K, Lazarova Z. (2005). Influence of enzyme aqueous source on RME-based purification of α-amylase. *Separation and Purification Technology*, 47, 43-51.
Quina, F.H.; Chaimovich, H. (1979). Ion-exchange in micellar solutions. 1. Conceptual-framework for ion-exchange in micellar solutions. *Journal of Physical Chemistry*, 83, 14, 1844-1850.
Brochsztain, S., Berci, P., Toscano, V.G., Chaimovich, H., Politi, M.J. (1990). Ion binding and selectivity in zwitterionic micelles. *Journal of Physical Chemistry*, 94, 17, 6781-6785.
Sarubbo, L.A., Oliveira, L.A., Porto, A.L.F., Duarte, H.S., Carneiro-Leão, A.M.A., Lima Filho, J.L., Campos-Takaki, G.M., Tambourgi, E.B. (2000). New aqueous two-phase system based on cashew-nut tree gum and poly(ethylene glycol). *Journal of Chromatography, B*, 743, 1/2, 79-84.