Ultrasonics Sonochemistry 31 (2016) 532–538

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

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

Purification of serratiopeptidase from *Serratia marcescens* NRRL B 23112 using ultrasound assisted three phase partitioning

Swapnil V. Pakhale, Sunil S. Bhagwat *

Department of Chemical Engineering, Institute of Chemical Technology, Matunga (E), Mumbai 400019, India

ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 27 January 2016
Accepted 29 January 2016
Available online 30 January 2016

Keywords:
Ultrasound assisted three phase partitioning (UATPP)
Serratiopeptidase
Serratia marcescens NRRL B 23112
Three phase partitioning (TPP)
Purification

ABSTRACT

The ultrasound assisted three phase partitioning (UATPP) is a novel bioseparation method for separation and purification of biomolecules. In the present work, UATPP was investigated for the first time for purification of serratiopeptidase from *Serratia marcescens* NRRL B 23112. Effect of various process parameters such as ammonium sulphate saturation, t-butanol to crude extract ratio, pH, ultrasonic frequency, ultrasonic intensity, duty cycle and irradiation time were evaluated and optimized. The optimized conditions were found to be as follows: ammonium sulphate saturation 30% (w/v), pH 7.0, t-butanol to crude ratio 1:1 (v/v), ultrasound frequency 25 kHz, ultrasound intensity 0.05 W/cm^2, duty cycle 20% and irradiation time 5 min. The maximum purity and recovery obtained from UATPP was 9.4-fold and 96% respectively as compared to the three phase partitioning (TPP) (4.2-fold and 83%). Also the process time for UATPP was significantly reduced to 5 min from 1 h as compared to TPP. The results indicate that, UATPP is an efficient technique for the purification of serratiopeptidase with maximum purity, recovery and reduced processing time.

1. Introduction

Serratiopeptidase is an extracellular metalloprotease enzyme isolated from *Serratia marcescens* (strain E-15). It contains 450 amino acids cleaved with the peptide bond having a molecular weight of approximately 50 kDa [1]. The purified serratiopeptidase has been extensively used all over in Europe and Asia as a potent analgesic and anti-inflammatory drug. Serratiopeptidase induces fibrinolytic, anti-inflammatory and anti-edemic (prevents swelling and fluid retention) activity to number of tissues, and its anti-inflammatory effects are superior to other proteolytic enzymes [2]. It is used to treat chronic inflammatory diseases, such as atherosclerosis, arthritis, bronchitis, carpel tunnel syndrome, fibrocystic breast disease, and sinusitis [3]. Fujisaki et al. proposed that, the oral serratiopeptidase can be used as an aid in the treatment or prevention of viral diseases, such as AIDS and hepatitis B and C [4].

The bacterium *S. marcescens* has been widely reported as a good producer of extracellular metalloprotease. Many researchers have worked on the purification and characterization of serratiopeptidase from different strains of *S. marcescens*, such as *S. marcescens* E-15, [5] *S. marcescens* ATCC 25419, [6] and *S. marcescens* NRRL B-23112 [7]. The purity of a therapeutic enzyme is of prime importance and hence purification plays a vital role in enzyme production. The success of any biochemical production depends on the development of cheaper processes, because downstream process is responsible for approximately 60% of the overall cost [8]. Several purification techniques have been reported in literature for the purification of serratiopeptidase such as, ammonium sulphate precipitation, acetone fractionation, ion exchange chromatography, gel permeation chromatography [9] and membrane separation, etc. Serratiopeptidase from *S. marcescens* NRRL B 23112 was purified by ammonium sulphate precipitation, acetone fractionation and ion exchange chromatography with AG 501-X8 mixed-bed resin to result in 5.7-fold purification with 51% recovery [7]. Decedue et al. have used ammonium sulfate precipitation and DEAE cellulose chromatography to achieve the 52% recovery of serratiopeptidase from *S. marcescens* ATCC 25419 [6]. These techniques are expensive, time consuming and difficult to scale up. So, it is desirable to develop a novel, more economic and environment friendly method to improve the recovery and purity of serratiopeptidase with lesser processing time [10].

Three phase partitioning (TPP) is one such novel approach for purification of enzyme and small molecules [11]. It involves an organic/aqueous system such as t-butanol and ammonium sulphate which is used to selectively extract the target molecule at the interface (in the form of precipitate) leaving the contaminants in the organic and aqueous phases. t-butanol is completely miscible with water, but upon addition of enough ammonium sulfate, the solution separates into two phases. The upper phase is

* Corresponding author.
E-mail address: ss.bhagwat@ictmumbai.edu.in (S.S. Bhagwat).

http://dx.doi.org/10.1016/j.ultsonch.2016.01.037
1350-4177/

organic and contains t-butanol, and the lower phase is the aqueous solution [12]. t-Butanol binds to the precipitated proteins, thereby increasing their buoyancy and causing the precipitates to float above the denser aqueous salt layer and forms the third phase (interphase) [13]. The actual mechanism of TPP is unfamiliar to research community; however osmolytic and kosmotropic effect, salting out, isotonic precipitation, cosolvent precipitation and protein hydration shifts have been postulated as key contributors for the partitioning of the target enzyme [14]. TPP has a unique ability to concentrate proteins from crude broths with higher purity than conventional concentration methods [13]. TPP is an inexpensive and easily scalable method and can be directly used with crude broth. TPP has been widely used to purify the therapeutic and industrial enzymes such as Nattokinase from *Bacillus natto* NRRL-3666 [13], β-galactosidase [15], invertase [11], and natural products like forskolin [16], ursolic acid [17]. In regard to purification of such biomolecules, this is the first report on the use of TPP for purification of serratiopeptidase. The partitioning of enzyme is a mass transfer driven phenomenon and any improvement in mass transfer leads to enhanced partitioning and subsequently purity of the target enzyme.

Ultrasound is a well recognized process intensification tool for extraction, fermentation, adsorption, aqueous two phase extraction, bioremediation and biocatalysis processes due to its enhanced mass transfer and yield [18,19]. TPP is also a mass transfer dependent phenomenon and the applicability of ultrasound on three phase partitioning has been studied to overcome the limitations of mass transfer in TPP. An ultrasonic wave leads to formation of gas filled bubbles. The subsequent collapse of these bubbles imparts vicious shock wave and mechanical shear to the surrounding environment. The change in local energy densities and mechanical shears incurred due to cavitational phenomenon accelerates the mass transfer across the different phases [20]. Substantial literature are available on the ultrasound assisted three phase extraction of various biomolecules such as ursolic acid, mangiferin, astaxanthin and fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 [21]. In spite of the considerable work on application of Ultrasound assisted three phase partitioning (UATPP), no literature pertaining to purification of serratiopeptidase using UATPP has been reported.

In the present work, an attempt has been made for the recovery of serratiopeptidase from fermentation broth of *S. marcescens* NRRL B 23112 using TPP and UATPP. The objective of the present work is to investigate a suitable method for enhanced recovery of serratiopeptidase. In the view of objective, various process parameters such as effect of salts and solvents, ammonium sulphate saturation, t-butanol to crude ratio, pH, ultrasonic frequency, ultrasonic intensity and duty cycle were studied and optimized. The novelty of the present work lies in the use of TPP and application of ultrasound to TPP (i.e. UATPP) for purification of serratiopeptidase from fermentation broth and to investigate the effect of medium chain alcohols on TPP.

2. Material and methods

2.1. Chemicals and reagents

All media components, Tris Buffer, Trichloroacetic acid (TCA), Phenylmethylsulfonyl chloride (PMSF), and Ethylenediaminetetraacetic acid (EDTA) were purchased from Himedia Ltd (Mumbai, India). Casein (Hammarsten grade) was purchased from Sisco Research Laboratory (SRL, Mumbai, India). Tert-Butanol and ammonium sulphate were purchased from S. D. Fine-Chem. Ltd. (Mumbai, India). All the chemicals used were of analytical grade.

2.2. Microorganisms

The culture of *S. marcescens* NRRL B 23112 was obtained from ARS Culture Collection, USA. It was maintained on a soybean casein digest agar medium and sub-cultured after every 15 days. The slants of *S. marcescens* NRRL B 23112 were stored at 4 °C in refrigerator.

2.3. Serratiopeptidase production

Serratiopeptidase was produced from an optimized media by *S. marcescens* NRRL B-23112. The culture was grown on a soybean casein digest medium in 250-ml Erlenmeyer flasks for 14 h at 25 °C and 180 rpm. The production medium consists of maltose 4.5%, Soybean meal 6.5%, K_2HPO_4 0.8%, NaCl 0.5% (w/v), pH 6.0 and was inoculated with 1% v/v (2×10^8 cells/ml) cell suspension of *S. marcescens* NRRL B-23112. The inoculated flasks were kept for incubation at 28 °C for 48 h at 180 rpm. The broth was centrifuged with 6708 × g at 4 °C for 10 min, and the supernatant was used as crude for three phase partitioning [22].

2.4. Three phase partitioning (TPP)

The cell-free broth (5 ml) was saturated with weighed amount (0–20%) of ammonium sulphate followed by addition of t-butanol (5 ml). The mixture was mixed on a magnetic stirrer with magnetic needle of dimension 20 mm × 6 mm at 5000 rpm for 60 min, 25 °C and mixture was allowed to separate for 1 h at 30 °C in a separating funnel to bring about the formation of the three phases (upper organic phase, middle interfacial precipitate and lower aqueous phase). The upper t-butanol layer and the lower aqueous layer were removed using a micropipette. The interfacial precipitate layer (potentially containing protein) was collected and dissolved in 50 mM Tris–HCl buffer (pH 8.0) and analyzed for serratiopeptidase activity as well as total protein concentration.

2.5. Ultrasound treatment

Concentration and partial purification of serratiopeptidase was carried out by ultrasound assisted three phase partitioning (UATPP). The ultrasonic water bath used for the study had an internal dimension of 0.23 × 0.15 × 0.15 m (Model No. 6.51200 H, Dakshin, India Pvt. Ltd.) with 4 magnetostrictive transducers arranged in zigzag position at the bottom of ultrasonic bath. The glass vessel (flat bottom) used in the study had capacity of 250 mL with dimensions of 0.13 m height, 0.05 m diameter. It was placed 0.02 m above the bottom of ultrasonic bath. The position of glass vessel in ultrasonic water bath has been previously optimized [23]. This position was noted and maintained for all experiments. The ultrasonic water bath temperature was varied and controlled with the water recirculation system. Ultrasonic bath was operated at two different frequencies viz. 25 and 40 kHz with maximum US intensity of 0.1 W/cm^2 (i.e. US power 200 W). A particular frequency was chosen throughout the experiment via a selector switch. Autotransformer was used to vary the AC voltage to change the irradiated power output.

UATPP experiments was carried out in a glass vessel containing mixture of 10 mL of crude broth, 10 mL of t-butanol and calculated amount of ammonium sulfate (30% saturation) at pH 7. Ultrasound frequency selected for irradiation was 25 kHz with 0.1 W/cm^2 intensity (i.e. 200 W rated power) and 20% duty cycle. Irradiated mixture was then allowed to separate for 1 h in separating funnel and the separated interphase was analyzed for enzyme activity and protein content. One factor at a time approach was used for optimization of operational parameters (i.e. broth to t-butanol ratio,