Protein Expression and Purification 133 (2017) 8–14

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Purification and characterization of a collagenase from *Penicillium* sp. UCP 1286 by polyethylene glycol-phosphate aqueous two-phase system

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ARTICLE INFO

Article history:
Received 11 January 2017
Received in revised form
22 February 2017
Accepted 22 February 2017
Available online 24 February 2017

Keywords:
Collagenolytic enzyme
Collagen
ATPS
Purification

ABSTRACT

Collagenases are proteolytic enzymes capable of degrading both native and denatured collagen, reported to be applied in industrial, medical and biotechnological sectors. Liquid-liquid extraction using aqueous two-phase system (ATPS) is one of the most promising bioseparation techniques, which can substitute difficult solid-liquid separation processes, offering many advantages over conventional methods including low-processing time, low-cost material and low-energy consumption. The collagenase produced by *Penicillium* sp. UCP 1286 showed a stronger affinity for the bottom salt-rich phase, where the highest levels of collagenolytic activity were observed at the center point runs, using 15.0% (w/w) PEG 3350 g/mol and 12.5% (w/w) phosphate salt at pH 7.0 and concentration. The enzyme was characterized by thermal stability, pH tolerance and effect of inhibitors, showing optimal collagenolytic activity at 37 °C and pH 9.0 and proved to be a serine protease. ATPS showed high efficiency in the collagenase purification, confirmed by a single band in SDS/PAGE, and can in fact be applied as a quick and inexpensive alternative method.

1. Introduction

Collagen is a protein composed of three peptide chains associated in a triple helical structure, found in connective tissues of animals and corresponding to about 30% of the entire protein content in the human body [1,2]. Collagenases are proteolytic enzymes capable of degrading both the native and denatured collagen [3–5] and they are related to physiological and pathological processes, with many applications in industry, medicine and biotechnology, as collagen hydrolysis, wound repair and production of bioactive peptides [5–9].

Among several methods described in the literature for separation and purification of proteases, ultrafiltration, precipitation and chromatography are the most common used [10]. However, for industrial scale, such procedures are not considered profitable due their high cost and long processing time [11]. Thus, the search for alternative protease purification methods has become increasingly common [12]. Liquid-liquid extraction using aqueous two-phase systems (ATPS) is one of the most promising bioseparation techniques which can, although being simple and inexpensive, be used in place of difficult solid-liquid separation processes, in the initial or subsequent purification steps [13].

Aqueous two-phase system has potential industrial applications because its: environment-friendly; low cost, processing time and power consumption; capacity of continuous operation and ease of scaling-up. Composed by a mixture of two polymers or a polymer

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http://dx.doi.org/10.1016/j.pep.2017.02.010
1046-5928/

and a salt (constituting two aqueous phases), ATPS has been used in bioprocesses which aim to recover and purify many biological products including proteins, genetic material, bionanoparticles, cells and organelles [12,14–16].

ATPS has been used to partition and recover several molecules as: human antibodies [17], catalase [18], clavulanic acid [19,20], polygalacturonases [21], citrinin [22], pepsin [23], bromelain [24,25], tannase [26], xylanase [27], lipase [28–30], elastase [31], alkaline protease [12], laccase [32], fibrinolytic proteases [33,34] and others. ATPS formed by PEG and phosphate has been described in collagenase extraction due PEG's favorable physical properties, particularly as regards viscosity and differences in density between the phases [7,13].

The present work aims to extract, purify and characterize a collagenase produced by *Penicillium* sp. UCP 1286, isolated from Caatinga's soil (Pernambuco – Brazil), and determine the best conditions for purification by ATPS. For this purpose, a 2^4 full factorial design was applied to determine the optimal levels of PEG molar mass, pH, phosphate salt and PEG concentrations.

2. Materials and methods

2.1. Microorganism

Penicillium sp. strain (UCP 1286) isolated from the soil of Serra Talhada, PE, Brazil (Caatinga biome) was obtained from UCP - Catholic University of Pernambuco Collection (UNICAP).

2.2. Culture medium

Malt Extract Agar was used as maintenance medium composed by: malt extract (0.5%), peptone (0.1%), glucose (2%) and agar 1.5 (%). The preparation of culture medium followed the methods of Lima et al. [35], with modifications, composed by: gelatin (0.5% w/v), $MgSO_4 \cdot 7H_2O$ (0.025 w/v), K_2HPO_4 (1.5 w/v), $FeSO_4 \cdot 7H_2O$ (0.015 w/v), $CaCl_2$ (0.025 w/v) and mineral solution (1% v/v).

2.3. Azocoll assay for collagenolytic enzyme activity determination

Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO) assay was carried out according to a modified version of the method developed by Ref. [36]. The absorbance of supernatant was measured at 520 nm by a UV-Vis spectrophotometer (model B582; Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per milliliter, necessary to increase the absorbance by 0.1, because of the formation of azo dye-linked soluble peptides.

2.4. Protein measurement

Protein concentration was determined by the method of Bradford [37] modified by the use of "Coomassie Blue Bright G-250" to bind proteins. The calibration curve was obtained from stock solutions of bovine serum albumin (BSA).

2.5. Aqueous two-phase system preparation

A phosphate buffer solution (PBS, 40% w/w) was prepared according to Lima et al. [7], at room temperature (25 ± 1 °C). Subsequently, PEG solutions with different molar masses, specifically 1500, 3350 and 8000 g/mol, were dissolved (60% w/w) in PBS and transferred to 15-mL graduated tubes. Aliquots of the fermented broth corresponding to 20% (w/w) of the total mass of system were later added, along with enough water to give a 10 g system. After 1 min of vortex shaking, the two phases were separated by settling for 120 min. The volume of each phase was then measured, and the protein concentration and collagenase activity were determined. To avoid interference of PEG and phosphate salt, all the samples were compared with protein-free standard solutions, with the same phase composition.

2.6. Experimental design

A 2^4 full factorial design was utilized to evaluate the influence of four independent variables, namely PEG molar mass (x_1), PEG concentration (x_2), pH (x_3), and phosphate salt concentration (x_4) on the parameters of partition coefficient, activity yield and purification factor of the collagenolytic enzyme. The experimental design included 16 runs and 4 repetitions at the central point, which were necessary to calculate the pure error (Table 1). A linear regression model was employed to predict the response, according to eq. (1):

$$R = b0 + \sum bixi + \sum bjxj + \sum bijxix \quad (1)$$

where b0 is the interception coefficient, bi and bj are the linear coefficients, bij are the interaction coefficients and xi and xj are the independent variables. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA); the first-order model equation was determined by Fischer's test. The experimental and predicted values were compared and the developed model validated with Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

2.7. Determination of the partition coefficient, activity yield and purification factor

The collagenase partition coefficient was determined as the ratio of the collagenase activity in the top phase (A_T) to that in the bottom phase (A_B):

$$K = \frac{A_T}{A_B}$$

The activity yield was defined as the ratio of A_B to the initial activity in the fermentation broth (A_F) and expressed as a percentage:

$$Y = \left(\frac{A_B}{A_F}\right) \times 100$$

The purification factor was calculated as the ratio of the specific activity in the bottom phase (A_B/C_B) to the initial specific activity in the fermentation broth before partition (A_F/C_F):

$$PF = \frac{\frac{A_B}{C_B}}{\frac{A_F}{C_F}}$$

where C_B and C_F are the protein concentrations, expressed in mg/

Table 1
Factor levels of the 2^4-full factorial design used to investigate collagenase partition and purification by ATPS.

Factor	Level		
	Low (−1)	Center (0)	High (+1)
PEG molar mass (M_{PEG})	1500	3350	8000
PEG concentration (C_{PEG})	12.5	15.0	17.5
Phosphate concentration (C_{PHOS})	10.0	12.5	15.0
pH	6.0	7.0	8.0