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A sustainable affinity partitioning process to recover papain from *Carica papaya* latex using alginate as macro-ligand

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ABSTRACT

The role of the natural biodegradable polymer alginate (ALG) as affinity macro-ligand for papain (PAP) was evaluated in order to design a new sustainable two-phase affinity strategy for recovering this enzyme from *Carica papaya* latex.

In presence of PAP, decreased values of intrinsic viscosity and hydrodynamic radius of ALG were observed. These results suggested a neutralizing effect of PAP on the polymer negative charges which caused a decrease of intra-chain repulsion forces in ALG molecules and a consequent shrinkage of the polymer size. Calorimetric measurements demonstrated a cooperative interaction between PAP and ALG which was enthalpy-entropically driven.

When partitioned *C. papaya* latex into aqueous two-phase systems (ATPSs) formed by polyethylene glycol (PEG) of MW 8000 and the biodegradable salt sodium citrate pH 5.20, only 20% of PAP was recovered at the PEG-enriched phase with a purification factor (PF) of 2.48. The addition of ALG 0.1% (w/w) into the system doubled the PAP partition coefficient ($K_{p,\ PAP}$), showing the ability of this polymer to enhance the enzyme recovery at the PEG-enriched phase and therefore, increasing the extraction efficiency. The subsequent addition of calcium chloride at a final concentration of 80 mM allowed the precipitation of the target enzyme and the recovering of PEG phase for recycling. The overall process showed a PAP recovery of 72% and a PF of 2.41. The proposed strategy not only conserved all the advantages of the reported extractions with PEG/ammonium sulfate (or phosphate) ATPSs, i.e., low cost, scalability and integration of the clarification and extraction steps, but also allowed overcoming their main drawbacks, i.e., the separation of the target molecule from the phase-polymer and the environmental impact caused by the disposal of these salts.

1. Introduction

Proteases play an important role in industrial processes due to their ability of transforming materials, i.e. protein structure and functionality, with high rate at sustainable conditions. Most commercialized proteases are those coming from microbial sources. However, some plant cysteine proteases such as papain (PAP) are still preferred and currently used for certain industrial applications due to its specificity and stability properties [1,2]. Papain (EC3.4.22.2) is a cysteine endopeptidase present in the latex of papaya fruit which has been extensively used in food industries as meat tenderizer, for stabilizing and chill-proofing beer and in baking processes. Besides, it has been applied in textile and pharmaceutical industries, and also for effluent treatments [3].

Currently, PAP is recovered from its natural source by a given combination of unit operations such as clarification, precipitation with salts, extraction with solvents, chelating or reducing agents, chromatographic techniques [4], filtration and spray-drying. Environmental impact, cost of resins, time-consuming processes, occupational allergies and unstable yielding products are the main disadvantages reported for the above mentioned downstream processes [2]. Alternative methods tending to avoid or minimize these difficulties have been developed.

Extraction with aqueous two-phase systems (ATPSs) is a methodology that integrates the clarification, concentration and purification of the target product in one unit operation. It exhibits many advantages including low cost, easy scalability and mild

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conditions to preserve biological molecules. Besides, it does not require sophisticated equipment [5] thus becoming an attractive strategy for industrial purposes. Different polymer/salt ATPSs such as polyethylene glycol (PEG)/phosphate [6] and PEG/ammonium sulfate [2,4] have been earlier reported for the extraction of PAP from papaya latex. Although the high PAP recoveries obtained with these systems, close to 90%, they presented two main drawbacks: the difficulty to isolate the extracted molecule from the polymer phase by back extraction and the use of high salt concentrations which becomes critical at process-scale and requires of disposal and corrosion considerations. Particularly, the use of ammonium sulfate and phosphates for any manufacturing process is discouraged since its discharge into water bodies can result in the promotion of algal growth and subsequent creation of anaerobic conditions. The treatment and disposal of wastes, required to minimize their impact, increase the downstream processing costs and consequently decrease their economic viability. In this context, the design of extracting protocols with ATPSs formed by biodegradable and/or recyclable components, would be desirable [7–9].

Affinity partitioning using smart macro-ligands is a powerful method used for separation of biomolecules [10,11] which integrates two-phase partitioning and affinity precipitation. The affinity macro-ligand is incorporated into the PEG/salt ATPS with the aim of directing the partitioning of the target molecule to the polymer-rich phase. Later, the precipitation of the ligand-biomolecule complex with an appropriate stimulus – pH, temperature, presence of certain ions – allows recovering both, the target molecule (PEG-free) and the phase polymer (PEG) which can be reused in a new extraction cycle. Affinity ligands are considered suitable for this purpose when they partition unevenly to the PEG phase, bind selectively/reversibly to the target biomolecule and can be precipitated/re-dissolved by changing medium conditions. Chitosan, Eudragit S-100 and alginate, among other polymers, fulfill these requisites [12], thus being widely used in these strategies [13]. Particularly alginate, a polysaccharide of marine origin, consisting of mannuronic (M-block) and guluronic (G-block) acid units has demonstrated to be appropriate to recover Bacillus acidopullulyticus pullulanase from a crude enzyme preparation [12], beta-amylase from sweet potato [14] and phospholipase D from peanuts [15]. Several reports have addressed on the ability of calcium alginate beads of immobilizing and stabilizing papain [16,17]. However, as far as we know, soluble sodium alginate (ALG) has not been used as bio-ligand for this protease in affinity strategies.

The aim of this work was to evaluate the suitability of ALG to be used as a macro-ligand for PAP in order to develop a new affinity partitioning process capable of recovering this protease from *Carica papaya* latex. The use of ALG in a PEG/sodium citrate (biodegradable salt) ATPS is expected to enhance the extraction of papain to the polymer-rich phase and allow its subsequent recovery by precipitation. The resulting strategy will allow overcoming the main disadvantages reported for the extraction with PEG/ammonium sulfate (or phosphate) ATPS such as the separation of the target molecule from the phase-polymer and the environment impact associated to the disposal of these salts.

2. Materials and methods

2.1. Chemicals and raw materials

Polyethylene glycols of different molecular weights (PEG600, PEG1000, PEG2000, PEG4600 and PEG8000), alpha-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and sodium alginate (ALG) of low viscosity, composed primarily of mannuronic acid residues, were purchased from SIGMA-Aldrich (USA). All other chemicals were of analytical grade. Commercial papain (PAP_{com}) from *C. papaya* latex was also purchased from Sigma Chem. Co. Stock solutions (4000 μM) of this protease in 100 mM sodium phosphate buffer pH 6.00 were gravimetrically prepared and kept at −18 °C until use. Stock solutions of ALG 2% (w/w) and PEGs 30% (w/w) were prepared by dissolving appropriate quantities of polymer in distilled water.

Fresh latex was collected from locally grown *C. papaya* trees. After washing the fruits with distilled water, several vertical incisions were made along the surface with a knife to a depth of 2–3 mm. The exuded latex was collected into a glass beaker set on ice. After collection, the latex was fractionated and stored at −18 °C. Thawed latex was dissolved in phosphate buffer pH 6.0 and the obtained suspension was referred to as "crude papain extract" (CPE). Its content of papain (PAP_{latex}), reducing sugars (RS) and total proteins (TP) was determined as described below.

2.2. Procedures

2.2.1. Partitioning measurements

2.2.1.1. Preparation of aqueous two-phase systems. Aqueous two-phase systems (ATPSs) with a final mass of 2 g were prepared by weighing appropriate quantities of stock solutions of PEGs of different molecular weight 30% (w/w), sodium citrate (NaCit) 25% (w/w) pH 5.20 and distilled water. The sodium citrate stock solution was prepared from a citric acid solution whose pH was adjusted to 5.20 by the addition of appropriate quantities of sodium hydroxide. At the mentioned pH, acid and base conjugate forms of citric acid coexist ($pKa_{2\ citric\ acid} = 4.76$) thus resulting in a mixture with buffer capability.

Table 1 shows the tie line length (TLL), the total and the phase compositions of the selected systems. ATPSs containing ALG as affinity macro-ligand were prepared by including appropriate amounts of ALG stock solution in the above mentioned systems to reach the desired ALG total final concentration of 0.1% (w/w). In this case, water content was reduced appropriately to keep the PEG and NaCit total concentrations unaltered.

2.2.1.2. Determination of partition coefficients. Partitioning behavior of papain (PAP_{com} and PAP_{latex}), sodium alginate (ALG), reducing sugars (RS) and total proteins (TP) in the assayed ATPSs were evaluated through their partitioning coefficients ($K_{p,\ PAPcom}$; $K_{p,\ PAPlatex}$; $K_{p,\ ALG}$; $K_{p,\ RS}$; $K_{p,\ TP}$) defined as:

$$K_{p,M} = \frac{[M]_{top}}{[M]_{bottom}} \quad (1)$$

where $[M]_{top}$ and $[M]_{bottom}$ represent the concentration of the partitioned molecule (M): PAP_{com}, PAP_{latex}, ALG, RS and TP at both top and bottom phases after reaching the equilibrium condition.

The procedure consisted of dissolving a given amount of either stock solutions (ALG, PAP_{com}) or crude papain extract (CPE) into the

Table 1
Total, bottom and top compositions of ATPSs formed by PEGs of different molecular weights and NaCit pH 5.20.

PEG molecular weight	TLL % (w/w)	Total composition % (w/w)		Bottom composition % (w/w)		Top composition % (w/w)	
		PEG	NaCit	PEG	NaCit	PEG	NaCit
600	48.37	21.00	16.20	0.77	27.89	42.65	3.69
1000	26.42	15.92	13.97	5.10	19.01	28.87	7.47
2000	41.44	13.75	14.40	0.13	21.59	36.78	2.24
4600	24.98	11.70	9.65	0.88	14.18	23.92	4.53
8000	24.42	11.69	9.52	1.44	13.52	24.19	4.63

TLL, tie line length.