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Aqueous two-phase systems: A simple methodology to obtain mixtures enriched in main toxins of *Bothrops alternatus* venom

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ABSTRACT

Phospholipase A2 (PLA_2) and protease (P) are enzymes responsible of myotoxic, edematogenic and hemostasis disorder effects observed in the envenomation by *Bothrops alternatus* pitviper. Their partitioning coefficient (Kp) in different polyethyleneglycol/potassium phosphate aqueous two-phase systems (ATPSs) was determined in order to both achieve a better understanding of the partitioning mechanism and define optimal conditions for toxin isolation. Polyethyleneglycols (PEGs) of molecular weights 1000; 3350; 6000 and 8000; different temperatures (5, 20 and 37 °C) and phase volume ratios of 0.5; 1 and 2 were assayed. PLA_2 partitioned preferentially to the top phase while P mainly distributed to the bottom phase. Either entropically- or enthalpically-driven mechanisms were involved in each case (PLA_2 and P). The aqueous two-phase system formed by PEG of MW 3350 (12.20% wt/wt) and KPi pH 7.0 (11.82% wt/wt) with a volume ratio of one and a load of 1.25 mg of venom/g of system showed to be the most efficient to recover both enzymes. It allowed obtaining the 72% of PLA_2 in the top phase with a purification factor of 2 and the 82% of P at the bottom phase simultaneously. A further adsorption batch step with DEAE-cellulose was used to remove satisfactorily the PEG from the top phase and recover the active PLA_2.

The proposed methodology is simple, inexpensive, and only requires professionals trained in handling basic laboratory equipment. It could be easily adoptable by developing countries in which the snakebite accidents cause considerable morbidity and mortality.

1. Introduction

Envenoming resulting from snakebites is considered by the World Health Organization one neglected tropical disease which represents an important worldwide public health concern, particularly in developing countries of Africa, Asia and Latin America [1]. The victims of snakebites are mostly young —agricultural workers and children—, therefore, the consequent economic impact is considerable. A timely administration of a specific antiserum has been demonstrated to be an effective tool; however, the poor access to health services and the scarcity of adequate antivenomits in the mentioned countries leads to poor outcomes and considerable morbidity and mortality [2].

Snake venoms are complex mixtures of different biomolecules such as peptides, proteins and enzymes, some of which are responsible for their toxic effects [3,4]. Antivenoms are usually obtained by immunizing host animals —horses, rabbits, sheeps— with successive inoculations of increasing amounts of the whole lethal venom. Prolonged immunization plans are required to avoid damaging effects in animals and to obtain efficient antisera. An alternative strategy, that includes a pre-immunization of horse with phospholipase A_2 —one of the main toxic components of *Crotalus durissus terrificus*— has been proposed by Fusco et al. [5]. This protocol demonstrated to avoid both impairing animal health and improving the antiserum neutralizing efficacy. More recently, we observed that shorter immunizing protocols, milder toxic effects and antisera with high neutralizing ability were also obtained by inoculating animals with the whole venom enriched in

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its predominant toxins [6].

In this context, the availability of appropriate antigenic mixtures containing the main venom toxins associated with damaging or lethal effects is an important requirement in manufacturing more specific antibodies. In addition, it is a good starting point in developing vaccines [7] and sensitive immunoassays for snake venom detection in medical diagnostic [8].

The composition, immunogenicity and effects of venom vary intra and inter species, thus depending on the geographical distribution and the age of the specimens. Venom toxins to include in a venom pool used for animal immunization should be selected on the basis of the geographical region where the anti-venom is intended to be distributed [7].

The pitvipers inhabiting Central and South America belong to the genus *Bothrops*. Particularly, *Bothrops alternatus* is a species widespread in Brazil, Paraguay, Uruguay and Argentina, whose bites create severe local tissue damage, local and systemic hemorrhagic effects. Among its components, the phospholipases A_2 and the metalloproteases have been reported to play primary roles in myotoxic, edematogenic and hemorrhagic effects observed in the envenomation by this pitviper [9–12]. The complexity of the crude venom makes it difficult to separate mixture. A multistep separation consisting of gel filtration, ion-exchange and reversed phase high-pressure liquid chromatography is usually needed [13,14] to obtain an enzyme of desirable purity. This requires resources scarcely availably in developing countries or rural regions such as advanced equipment –columns, pumps and matrix– and professionals trained in special techniques.

Liquid–liquid extraction with aqueous two-phase systems (ATPSs) has proved to be a powerful tool for separating and purifying mixtures of biomolecules [15,16]. Culture media and crude extracts can be directly loaded into the ATPSs without a previous centrifugation or filtration step [17]. This technique exhibits several advantages such as simplicity, short processing times and low cost [18]. Furthermore, this process does not requires of sophisticated equipment and its scale-up is easy and reliable [19]. The ATPSs have been used in a wide range of biotechnological applications such as the recovery of recombinant proteins in corn [20], the purification of human antibodies [21] and Plasmid DNA [22] and the extraction of trypsin and chymotrypsin from bovine pancreas [23,24].

Protein partitioning and selectivity of the ATPS extraction depends on factors such as the phase-forming salt and polymer, the volume ratio (Vr) of phases and the sample load. The relation between the partition coefficient (Kp) of a target biomolecule and the ATPS parameters must be evaluated previously to develop an appropriate extraction protocol.

The goal of this work was to explore those variables that affect the partitioning equilibrium of phospholipase A_2 and protease toxins present in *B. alternatus* venom in order to optimize the extraction and develop a strategy capable of recovering venom mixtures enriched in the mentioned toxins. These toxins represent more than 50% of total venom protein [25] and are responsible of the main local and systemic effects caused in snakebites victims. At the same time, this information could contribute to a better understanding of the mechanisms involved in protein partition in ATPSs.

2. Materials and methods

2.1. Chemicals

Polyethyleneglycols of average molecular masses 1000; 3350; 6000 and 8000 (PEG1000, PEG3350, PEG6000, PEG8000) were purchased from Sigma Chem. Co. and used without further purification. All other reagents were of analytical quality.

Pooled crude venom of *B. alternatus* pitvipers was obtained from the Serpentarium of the local zoo in Corrientes, Argentina. It was lyophilized and then, stored at −20 °C until being required. Before using, the venom was diluted with phosphate buffered saline solution (PBS) pH 7.2, the resultant suspension (50 mg lyophilized venom/mL PBS) being applied for studies.

2.2. Experimental

2.2.1. Partition measurements

2.2.1.1. Preparation of the aqueous two-phase systems. Stock solutions of the phase components: potassium phosphate (KPi) pH 7.0 (30% w/w), solid PEG –of different molecular weights– and water were mixed in order to prepare the two-phase aqueous systems (total mass 20 g). The total system compositions selected from the binodal diagrams in literature [18] are shown in Table 1. After a thorough gentle mixing of the system components, low speed centrifugation was used to favour the phase separation. Phases were withdrawn and used to reconstitute several two-phase systems, in which the venom partitioning behaviour was evaluated.

2.2.1.2. Determination of the partition coefficients (Kps) of phospholipase A_2 and protease. Partitioning behaviour of phospholipase A_2 (*PLA_2*) and protease *(P)* was analysed by dissolving a given amount of venom suspension (50 μL) into two-phase systems of total mass 2 g with different ratios of top/bottom phase-volumes (0.5; 1 and 2). After mixing by gentle inversion for 10 min and leaving it to settle for at least 60 min, each system was centrifuged at low speed for the two-phase separation. These experiments were carried out in graduated tubes in order to appreciate the top/bottom phase-volumes. No volume-changes were observed after partitioning. Appropriate aliquots from separated phases were taken and diluted conveniently to determine the content of PLA_2 and P through activity measurements. The content of total protein (TP) in each phase was also determined according to the description bellow.

The partition behaviour of PLA_2, P and TP was evaluated by calculating the partition coefficient according to:

$$Kp = \frac{[protein]_{top\ \ phase}}{[protein]_{bottom\ \ phase}} \frac{f_{top}}{f_{bottom}} \quad (1)$$

The [protein] was replaced by enzyme activity when calculating the partition coefficient of PLA_2 and P. A correction factor (f_{top}, f_{bottom}), which comprises the effect of the components of a given phase (top/bottom) on the activity measurements, was included. It was calculated as follows:

Table 1
Total and phase compositions for PEG/Pi ATPSs.

Total composition (% wt/wt)		Top phase composition (%wt/wt)		Bottom phase composition (%wt/wt)	
KPi	PEG	KPi	PEG	KPi	PEG
PEG1000					
16.20	17.00	3.10	39.10	24.00	1.60
PEG3350					
11.82	12.20	5.56	23.90	17.41	1.30
PEG6000					
11.04	13.98	4.58	25.64	16.60	0.46
PEG8000					
10.90	15.00	3.10	33.00	15.59	1.60