

Microsphere entrapped bee-venom phospholipase A2 retains specific IgE binding capacity: a possible use for oral specific immunotherapy

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The only specific treatments of allergy are long and exacting desensitization by subcutaneous injections of the allergens. While oral administration of allergens could greatly facilitate these treatments, effective delivery systems are needed to prevent allergen degradation in the gastrointestinal tract and to enable their uptake by Peyer's patches. The potential for bee-venom phospholipase A2 (PLA2) to be used in such oral immunotherapy was tested. For this purpose, PLA2 potential alterations were analysed when encapsulated into poly(D,L-lactide-co-glycolide) microspheres by double emulsion solvent evaporation. It was shown that microencapsulation had only limited effects on the integrity of the entrapped PLA2, which retained its fully specific murine IgE binding capacity. Thus, PLA2 loaded microspheres could represent a potential delivery system for bee venom allergy-specific oral immunotherapy.

Keywords: Microspheres, IgE binding capacity, poly(D,L-lactide-co-glycolide), bee-venom phospholipase A2, allergen.

Introduction

Allergies represent the sixth leading pathology and their incidence is rapidly growing world-wide (Holt, 1998). Specific immunotherapy (SIT), the only specific therapy against allergy, consists of subcutaneous injections of increasing doses of allergen. Oral delivery systems could facilitate allergen administration for SIT and ease this long and exacting treatment. In order to be efficient for oral SIT, such delivery systems should protect the allergen against the harsh gastrointestinal environment and ensure the absorption of the bioactive agents in an unaltered form. In this regard, intact particles can be taken up by microfold (M) cells that cover intestinal Peyer's patches. It has been shown that M cells can internalize microspheres from the lumenal contents and expose them to immunocompetent cells in the gut-associated lymphoid tissues (Gilligan and Li Wan Po 1991, Haseto *et al.* 1994, Blanas *et al.* 2000). The poly(D,L-lactide-co-glycolide) (PLGA)

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copolymers have been widely studied as drug delivery systems because of their biodegradable and biocompatible characteristics (Brannon-Peppas 1995). Moreover, they protect protein against potentially deleterious gastric and gut secretions (Eldridge *et al.* 1990, Takahata *et al.* 1998). Numerous studies have highlighted the influence of microparticle diameter on the uptake by Peyer's patches. It appears that this uptake is limited to small microspheres of 10 μm diameter and lower. More specifically, the majority of microspheres smaller than 5 μm are transported through the efferent lymphatics within macrophages and are found in the spleen and lymph nodes. On the other hand, microspheres larger than 5 μm in diameter remained in the Peyer's patches (Eldridge *et al.* 1990). This process can lead to both systemic and secretory immune response after ingestion of microspheres of appropriate sizes (Mestecky *et al.* 1994).

The potential use of these copolymers was investigated as oral delivery systems for the PLA2 allergen. Here, it was reported that PLA2 can effectively be encapsulated into PLGA microspheres without alteration of its IgE binding capacity.

Material and methods

Microsphere preparation

Bee venom phospholipase A2 (PLA2) was encapsulated within PLGA microspheres using a double emulsion solvent evaporation method. The PLGA copolymer was obtained from Sigma and consisted of LA/GA = 75/25, molecular weight 90 000–126 000. The copolymer was dissolved in HPLC grade methylene chloride to obtain 16 ml of a 10% w/v solution. PLA2 (Sigma) was dissolved in NaCl 0.15 M to obtain an 8 mg/ml solution. One millilitre was added to the PLGA solution and the mixture was then emulsified with an Ultra-turrax at 13 500 rpm/min for 1 min. The resulting water in oil (w/o) emulsion was added to 200 ml of an aqueous 1% polyvinyl alcohol (Mowiol 40/88, Aldrich) solution and then emulsified for 1 min with Ultra-turrax at 13 500 rpm/min. The resulting w₁/o/w₂ emulsion was mechanically stirred for 4 h at room temperature under pulsed air flow to achieve complete evaporation of methylene chloride. Microspheres were harvested by centrifugation (10 min, 200 g). Non-encapsulated PLA2 was removed by three washes with distilled water. Microspheres were then collected, freeze-dried and stored at +4 °C in the presence of silica gel. The mean diameter of microspheres, determined using a Coulter counter (Coultronics), was $4.6 \pm 0.2 \mu\text{m}$. Protein content, determined by microBCA (Pierce) after PLA2 extraction (see below), was 50 μg of PLA2 per 100 mg of microparticles.

Immunization and PLA2-specific IgE assay

PLA2-specific murine IgE were obtained after immunization of FVB mice (Iffa Credo, France) with four intraperitoneal injections of 0.6 μg of PLA2 adsorbed on 2 mg of aluminium hydroxide. Suspensions were prepared in 0.15 M NaCl solution and mice were injected with 100 μl . Sera from five mice were collected by retro-orbital puncture after the last immunization and pooled. A pool of sera from five non-sensitized (naive) FVB was also constituted. Pools were sampled and stored at -20 °C until PLA2-specific IgE determination.

The PLA2-specific IgE levels were determined by ELISA. For this purpose, microtiter plates (Nunc-maxisorp) were coated with PLA₂ (4 µg/ml in PBS pH 7.4) and incubated for 24 h at 4 °C. After washing with PBS containing 0.05% Tween (PBST), plates were blocked for 1 h at 37 °C with 3% bovine serum albumin (BSA) in PBS. Plates were then washed with PBST and incubated for 24 h at 4 °C with dilutions of serum samples. Another PBST wash was performed before addition of the biotinylated anti-IgE monoclonal antibody (Pharmingen 02122D) at 2 µg/ml, and incubated for 1 h at 37 °C. This step was followed by a wash with PBST and incubation with streptavidin-peroxidase (1:5000 dilution, Sigma) for 30 min at 37 °C. After a last wash, the reaction was revealed with the colorimetric substrate orthophenylene diamine (0.5 mg/ml) in 0.05 M sodium citrate buffer pH 5.1 and H₂O₂ (30%, 0.25 ml/l). The reaction was stopped by addition of 2 N H₂SO₄ and absorbance was read at 490 nm using a microplate reader (Bio-Tek Instruments). Sample, biotinylated antibody and streptavidin-peroxidase were diluted in PBS containing 1% BSA. Titers were determined as the inverse of the highest dilution which gave an absorbance equal to twice the absorbance given by the pool of sera from naive FVB mice.

Specific IgE binding capacity of extracted PLA2

The conservation of the specific murine IgE binding capacity of extracted PLA2 was studied by performing a competing Enzyme Linked Immuno Sorbent Assay (ELISA).

More specifically, PLA2 solutions of concentrations (determined by micro-BCA) ranging from 0–15 µg/ml were made in PBS containing 1% BSA. In this regard, the native commercially available PLA2 and the microsphere-extracted PLA2 were used for these competitive assays. PLA2 was extracted from loaded microspheres by dissolving 100 mg of microspheres in 1 ml of methylene chloride. Two millilitres of PBS were then added and the mixture was strongly shaken for 3 min. The resulting emulsion was centrifuged and the aqueous phase containing extracted PLA2 was removed.

For the competitive ELISA, native or microsphere-extracted PLA2 solutions were mixed in equal parts with the pool of PLA2-specific sera first diluted 1:25 with PBS containing 1% BSA. These mixtures were incubated for 1 h at 37 °C, they were then added in duplicate to microplates coated with native-PLA2 and blocked with BSA 3%. Titration of PLA2-specific IgE was performed by the ELISA assay described above.

After absorbance reading, the PLA2 concentration which induces a decrease of 50% of the absorbance induced by the serum alone, or inhibitory concentration 50 (IC50), was determined for each type of PLA2.

Results

Competition of, on the one hand, native PLA2 and, on the other hand, extracted PLA2 with native PLA2 for specific IgE binding allowed IC50 determination. Results are presented in figure 1. IC50 was calculated after log/log transformation, on the linear zone.

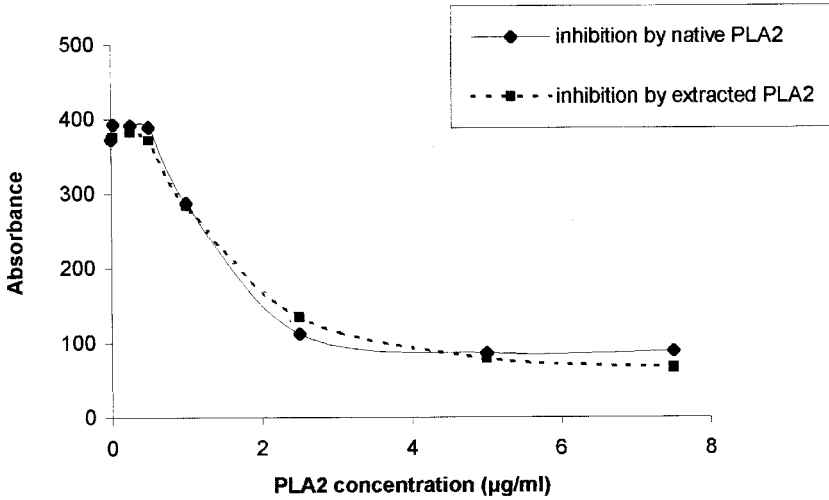


Figure 1. Assessment of specific IgE binding capacity of microspheres-extracted PLA2. Specific IgE titration was performed on 50/50 mixtures of, on the one hand sera and native PLA2, and on the other hand sera and extracted PLA2. The absorbance obtained by ELISA is reported for each PLA2 (native and extracted) concentration.

- When competition occurred between native PLA2 in solution and native PLA2 fixed on microplates, the resulting equation was:

$$\log(1000 \times \text{absorbance}) = -0.706 \times \log(\text{native PLA2 concentration}) + 2.399$$

- When competition occurred between extracted PLA2 in solution and native PLA2 fixed on microplates, the resulting equation was:

$$\log(1000 \times \text{absorbance}) = -0.672 \times \log(\text{extracted PLA2 concentration}) + 2.401$$

The $\log(1000 \times \text{absorbance}/2)$ calculated for the serum alone, without performing any mixture with PLA2 before its addition to microplates, was 2.271. Calculated IC50 were 1.5 and 1.6 µg/ml for native and extracted PLA2, respectively. Inhibition of 50% of native PLA2-IgE binding with extracted PLA2 required only 7% more PLA2 than if native PLA2 was used.

Discussion

The microparticle preparation method exposes the protein to potentially damaging conditions: exposure to organic solvent and high speed shear (Mestecky *et al.* 1994). As the desensitization process needs the integrity of protein epitopes, it was very important to check the integrity of these epitopes after entrapment. This experiment concerned extracted protein, that is to say PLA2 of the internal phase as well as PLA2 adsorbed on the microsphere surface. As B cells recognize discontinued epitopes constituted of amino acids non-adjacent in the primary sequence of the protein, three-dimensional structure is, therefore, essential for antigen recognition by immunoglobulins. The preservation of epitopes for IgE was studied. As the binding capacity of specific IgE with entrapped and then extracted PLA2 was preserved, one can conclude that epitopes for IgE were not altered in

their tertiary structure. The objective of this study was to produce biodegradable PLGA microspheres, without modification of the entrapped protein, to provide oral delivery system of allergen for specific immunotherapy. Prepared microspheres displayed optimal particle size for Peyer's patches uptake. The integrity of entrapped PLA2 was not affected as it retained its specific IgE binding capacity. As the microsphere preparation has minimal degradation effects on this allergen, PLGA microspheres would constitute an effective device for oral administration of bee venom PLA2.

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