

Immunochemical Characterization of Antigenic Fragments of *Olea europaea* and *Phleum pratense*

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Key Words

Olea europaea · *Phleum pratense* · Allergen fragments · Immunotherapy · T cell proliferation

Abstract

Background: The modulation of T cell responses by natural peptides is of potential use in the treatment of allergic diseases and reduces the risk of IgE-mediated reactions. The objectives of this study were to evaluate the allergenicity of depigmented, pepsin-digested fragments of *Olea europaea* and *Phleum pratense* extracts and their capacity to induce T cell proliferation. **Methods:** Lyophilized *O. europaea* and *P. pratense* extracts were depigmented following standardized methods, digested with pepsin and fractionated by ultrafiltration; a fraction containing components in the molecular weight range of 1–10 kD was selected. The blood of 20 allergic individuals was used to conduct T cell proliferation studies. **Results:** Pepsin-digested fragments of both pollen extracts lost more than 95% of their IgE-binding capacity by ELISA inhibition. Greater proliferation indexes than their respective native extracts were obtained in 55% of *O. euro-*

paea and in 95% of *P. pratense* allergic patients. **Conclusions:** This study demonstrates that pepsin-digested allergenic fragments of depigmented extracts can be produced in a standardized, reproducible manner. These fragments are significantly less allergenic and retain, or even increase, their capacity to induce T cell proliferations.

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Introduction

The induction of an immune response to foreign antigens requires the cooperation of various cell types, including antigen-presenting cells and T and B lymphocyte populations. It is well established that most undesirable side effects of allergen vaccines used in the treatment of allergic respiratory diseases are the result of IgE-mediated reactions. Therefore, reducing the allergenicity of allergen vaccines, while maintaining its reactivity with the other components of the immune system such as T cells, represents a sensible approach [1]. Antigenic proteins may possess determinants recognized separately by B and T cell

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populations [2] and some controlled processes may lead to the separation of these determinants, or to their selective degradation [3, 4].

Studies of ultraviolet spectrophotometry demonstrate that pollen extracts contain several groups of low-molecular-weight pigments, including flavonoids, which may occur as water-insoluble free flavonoids and water-soluble flavonoids-glycosides. The first group is largely removed during the defatting process and a great proportion of the second group is removed by dialysis during the production process. However, it can be shown by UV spectroscopy and thin-layer chromatography that a considerable proportion of the flavonoids remains firmly bound to dialyzed pollen proteins by physical forces [5]. Since flavonoids are known inhibitors of several cellular functions [6, 7], and of histamine release from mast cells and basophils [8], they may influence the *in vivo* and *in vitro* allergenicity and diagnostic accuracy of pollen extracts. Therefore, we decided to use depigmented allergen preparations to produce allergen fragments.

The enzymatic degradation of allergenic proteins creates many small immunoregulatory peptides that contain T cell epitopes with decreased IgE reactivity. These enzymatically derived allergen fragments induce T cell responses [9]. Strandberg et al. [10] reported that chymotrypsin treatment of rye grass pollen induced potent T cell, but not B cell responses, in a murine model. In a study with pepsin-derived fragments of BSA, the preserved T cell epitopes in such BSA fragments favored immune suppression rather than helper cell function. This suppression was mediated by T cells.

The clinical relevance of these enzymatically modified allergen extracts has been demonstrated [11, 12]. A pepsin-digested ragweed extract was compared with a crude ragweed extract in the treatment of ragweed-sensitive patients. Immediate skin test activity of the peptic fragments was 1,000-fold less than that of the original crude ragweed material. The peptic fragment mixture was more effective than the native extract in relieving clinical symptoms caused by ragweed. The group receiving peptide fragments reached a concentration of allergen injection 14 times higher than the group receiving crude ragweed [11]. Overall, this approach has been tested with rye grass [2–4, 10], peanut [9], ragweed [11, 12], phospholipase A₂ [13, 14], bovine serum albumin [15] and ovomucoid [16]. This study extends the experiments to *Olea europaea* and *Phleum pratense* and describes the characteristics of the obtained peptides.

Material and Methods

Allergenic Extracts

Dialyzed (nominal cut off 3 kD), lyophilized *O. europaea* and *P. pratense* pollen extracts were depigmented following standardized methods (European Patent EP 0 662 080 B1). Briefly, chromophoric substances physically adsorbed to nonmodified proteins were removed in controlled dialysis at pH 2. Depigmented extracts were digested with immobilized pepsin in 4% cross-linked agarose (Sigma) at pH 2 at 37°C and fractionated by ultrafiltration. The fragment fraction containing components in the molecular weight range of 1–10 kD was selected in each case.

Specific Antibody Binding

The lost of allergenic and antigenic potency was evaluated by ELISA inhibition experiments using specific IgE and IgG antibodies. Native and fragmented extracts were compared. Results are expressed as the amount of extract (ng) needed to produce 50% inhibition; two different serum pools were used, containing high titers of specific IgE to *P. pratense* and *O. europaea*, respectively.

Skin Tests

Skin tests were performed in 20 *O. europaea*- and in 6 *P. pratense*-sensitive individuals with clinical symptoms of rhinoconjunctivitis and/or asthma. Native, depigmented and fragmented extracts were used at a concentration of 1 mg/ml. Skin test reactions were measured by computerized papulometry and expressed as the mean $\pm$ SD of the areas (mm²).

T Cell Proliferation

Isolated peripheral blood mononuclear cells of 20 individuals with positive skin tests and clinical symptoms of allergic rhinoconjunctivitis and/or asthma were used to conduct T cell proliferation studies. Cellular proliferation was measured by ³H-thymidine incorporation. The proliferative responses were expressed as the stimulation index. These patients resided in a geographical area, where both allergens are widely distributed (Córdoba, Spain).

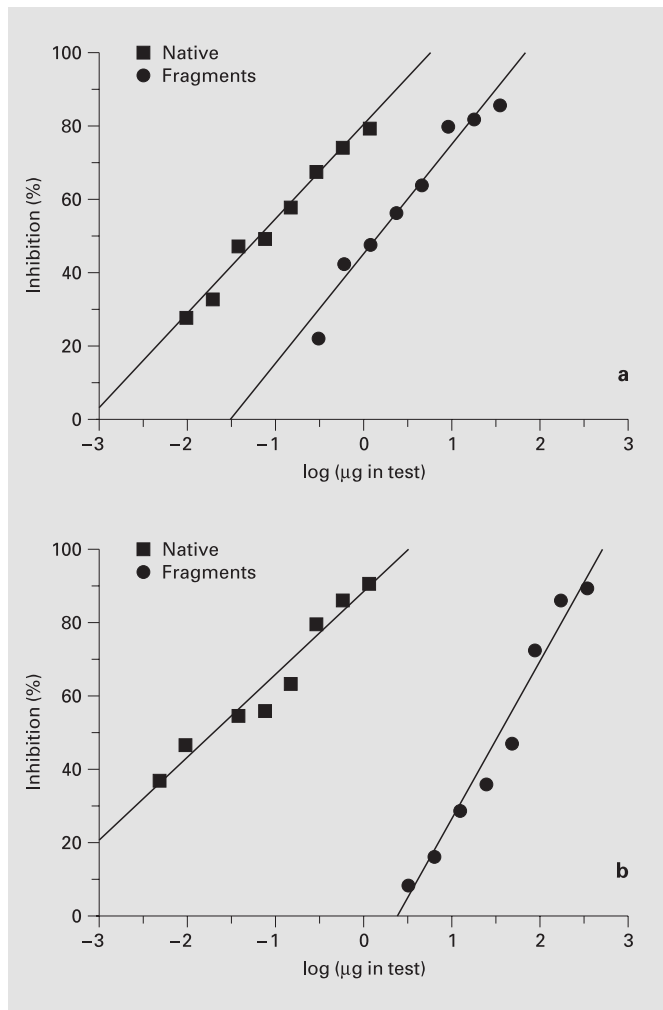
Acute Toxicity Studies

A method outlined earlier was followed [17]. It consisted of the intraperitoneal injection of 1 mg/ml into each of 5 healthy mice, weighing 17–22 g. The animals were observed for 7 days. None of them showed any signs of ill health after this period.

Results

Pepsin-digested fragments of both pollen extracts lost more than 95% of their IgE- and IgG-binding capacity (table 1). The results of the ELISA-specific IgE inhibition assays with *O. europaea*, *P. pratense* and their respective fragments are shown in figures 1a and b. Skin test results are shown in table 2. Figures 2a and b show the results of T cell proliferation studies; greater proliferation indexes were obtained with fragments than with their respective native extracts in 55% of *O. europaea*- and in 90% of *P. pratense*-allergic patients.

1



2

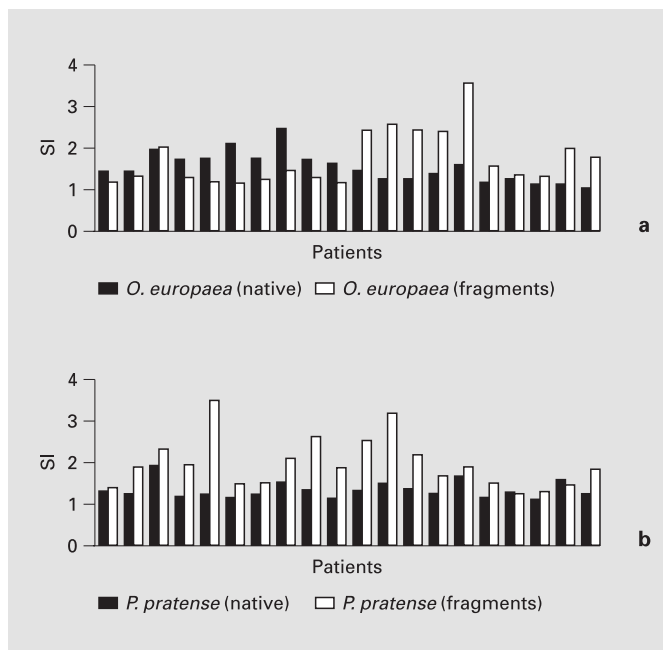


Table 1. Results of specific IgE and IgG inhibition experiments expressed as nanograms of lyophilized extract needed to produce 50% inhibition

Extract and type		EIA, ng	
		IgE binding	IgG binding
<i>Olea</i>	Native	66	23
	Depigmented	115	22
	Fragmented	1,550	948
<i>Phleum</i>	Native	19	53
	Depigmented	34	85
	Fragmented	38,985	47,122

Table 2. Skin test results

Allergen	Native	Depigmented	Fragmented
<i>O. europaea</i>	96.5 ± 32	93.8 ± 32	15.8 ± 20 (83% reduction)
<i>P. pratense</i>	66.6 ± 34	70.1 ± 38	18.3 ± 15 (72.5% reduction)

Discussion

Herein we describe the results of the immunochemical characterization of depigmented extracts of *P. pratense* and *O. europaea*, two species widely distributed in Spain and greatly allergenic. The fragmentation of the extracts, using immobilized pepsin, led to an extensive reduction of the reactivity with human antibodies. However, the ability of these preparations to induce a proliferative response by peripheral T lymphocytes from atopic patients was retained and, in some cases, even enhanced. The results obtained are similar to those obtained for other allergens, such as ragweed, which, in a clinical trial, demonstrated a significant clinical benefit.

This methodology generates products, which are closely similar to natural T cell epitopes. These fragments retain epitopes present in the natural pollen extracts,

Fig. 1. ELISA-specific IgE-binding experiments, using *O. europaea* (a) and *P. pratense* (b) native extracts in their respective solid phase. **Fig. 2.** Results of the T cell proliferation assays using T cells of *O. europaea* (a) or *P. pratense* (b) sensitive patients. SI = Stimulation index.

which often represent immunodominant sites, as demonstrated by the specific IgE inhibition assays. Such sites, which are retained in spite of depigmentation and enzymatic treatment, may be absent from synthetic peptides or recombinant allergens. We, therefore, conclude that the immunological properties present in fragmented allergens

are desirable for immunotherapeutic vaccines, due to their reduction in IgE reactivity, the absence of toxicity, the pattern of T cell recognition and a great safety margin. More in vitro studies, including other allergenic species, in vivo studies are needed to evaluate stability, clinical efficacy and safety.

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