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MALDI-TOF MS analysis of labile *Lolium perenne* major allergens in mixes

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Clinical and
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Allergy

Summary

Background It is well known that allergen extracts used for specific therapy of allergic disorders are commonly stored as mixtures, causing an alteration of its stability.

Objective The aim of this report is to identify pollen allergens susceptible to degradation during storage of mixtures containing different sources of proteases in the absence of glycerol as a preserving agent.

Methods Mixes containing *Lolium perenne* (*Lol p*) pollen extract with either *Aspergillus fumigatus* or *Periplaneta americana* extracts were prepared and co-incubated for 90 days at 4 °C. Samples were taken off at fixed times and comparatively tested by *in vitro* and *in vivo* assays with atopic patients. Selected pollinic allergens were subjected to MALDI-TOF MS analysis.

Results ELISA inhibition evidenced the loss of potency from ryegrass extract, and immunoblotting assays showed the degradation of specific pollinic allergens during storage of mixtures containing protease-rich sources. An *in vivo* intradermal skin assay confirmed the gradual loss of the biological activity of *L. perenne* pollen extract co-incubated with non-related protease-rich extracts in comparison with that of the control pollen extract.

MALDI-TOF MS analysis allowed us to determine that *Lol p 1* and *Lol p 5* are susceptible to proteolysis whereas *Lol p 4* was found to be resistant to degradation during storage.

Conclusions *Lol p 1* and *Lol p 5* degradation is responsible for the loss of the biological activity of *L. perenne* pollen extract when co-incubated with protease-rich fungal and cockroach extracts in the same vial for months in the absence of glycerol as a preserving agent. The integrity of these major allergens must be preserved to increase the vaccine stability and to assure efficacy when mixes are used for immunotherapy.

Keywords allergens, *Aspergillus fumigatus*, *Lolium perenne*, MALDI-TOF MS, *Periplaneta americana*, proteases

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Introduction

Treatment of IgE-mediated allergic diseases requires specific immunotherapy using standardized allergen preparations with known composition, potency and stability. However, in practice, many researchers tend to mix allergen extracts from different biologic materials in their immunotherapy studies, even though their composition is only partially known, to simplify the administration. Otherwise, allergenic potency can diminish during storage, after dilution or by mixture with allergen extracts containing proteases [1–4], and the presence of these enzymes in allergen extracts has been well docu-

mented [5–7]. Unfortunately, the loss in potency still affects the results of proper allergy diagnosis and immunotherapy.

The combination of pollinic cross-reacting allergens showed no differences compared with the use of single allergen extracts [8]. By contrast, mixing of non-related allergen extracts showed degradation of some pollen allergens that can cause a decrease in some pollens' allergenicity [2, 4]. *Periplaneta americana* and *Aspergillus fumigatus* allergen extracts contain a high content of proteases [5, 6] that can cleave their own proteins and pollen extract proteins in vaccines. *P. americana* extract, rich in cysteine and serine proteases [5], commonly used in mixes for immunotherapy, is

able to reduce the potency of different allergen extracts including some grasses [3]. The decrease in activity was presumed to be due to enzyme degradation of major grass allergens. In addition, the combinations of several pollen extracts with either *Alternaria*, *Cladosporium* or *Fusarium* species extracts, followed by storage led to a loss of pollen extract potency, and caused a strong degradation of individual pollen allergens in extract mixtures [3, 4]. Conversely, the *Penicillium* species extract did not show any effect on the allergenic potency of pollen allergens [3].

Pollen extracts contain, in addition to non-allergenic components, a series of relevant and other less important allergens. In grass pollen extracts, based on the prevalence of IgE reactivity among grass pollen-sensitized individuals, groups 1, 4 and 5 qualify as major allergens [9–11]. Group 4 homologues are present in grass extracts but to different extents [12]. By contrast, group 1 and group 5 allergens are present in most grass species showing immunochemical similarities between different grasses, but they present differences in concentrations among species [9, 13]. On the other hand, pollens also contain a variety of enzymes, including proteases. Birch pollen contains only traces of gelatinases and timothy pollen contains low amounts of metalloproteases at 80 kDa [4]. In addition, mass spectrometric analysis of electrophoretically separated allergens from some grass pollen diffusates showed peptidase and proteolytic activity, suggesting that pollen allergens analogous to other allergens may also be proteases [14].

The aim of this work is to identify the specific *Lolium perenne* (*Lol p*) pollen allergens susceptible to the effect of protease-rich extracts, responsible for the decrease in the biological activity of pollen, in therapeutic allergen mixes. For this purpose, *A. fumigatus* and *P. americana* extracts were separately mixed with *Lol p* pollen both in the presence or in the absence of glycerol, and the mixtures were stored at 4 °C for 90 days and analysed either by *in vitro* (SDS-PAGE, western blot, ELISA inhibition and gel filtration in an FPLC system) or *in vivo* (intradermal skin test assay) methods at different fixed times after mixing. We used MALDI-TOF mass spectrometry in combination with electrophoretic and immunologic techniques to identify specific pollen allergens susceptible to degradation or resistant to proteolysis during storage in mixes. A quantitative intradermal skin test measurement assay was used to highlight the clinical relevance of the deleterious effect due to proteolysis of specific major allergens when mixes are used for immunotherapy.

Material and methods

Preparation of allergen extracts

The *Lol p* pollen raw material (Hollister-Stier, Spokane, WA, USA) was extracted in phosphate-buffered saline (PBS), by stirring at 4 °C in 1:5 (w/v) ratios. After

centrifugation at 12 500 g for 40 min, the clarified extract was dialysed in PBS. *A. fumigatus* and *P. americana* extracts were prepared following the Frugoni-Hansen method described elsewhere [15]. Extracts were sterilized with Millipore filters (Bedford, MA, USA) (0.22 µm). Sterility controls were performed and the extracts were frozen and stored at –20 °C. Bradford's method was applied to determine the protein concentration of each extract, using bovine serum albumin as a standard solution [16].

Study design and preparation of allergen extract mixtures

The allergen extracts were diluted in sterile PBS to a concentration of 0.4 mg/mL. Mixtures were prepared by mixing equal parts of *Lol p* with either *A. fumigatus* or *P. americana* extracts. Single extracts and extract mixtures were stored at 4 °C for 90 days. At days 1, 7, 15, 30, 60 and 90, samples were taken and frozen at –70 °C for subsequent analysis. Test allergenic extracts consisted of *Lol p* mixed with *A. fumigatus* or *P. americana*. The control extract consisted of *Lol p* in PBS solution. Fifty percent glycerinated or non-glycerinated extracts of both mixtures were prepared. At each time-point, aliquots were withdrawn from each mixture vial for *in vitro* and *in vivo* analysis.

Patient eligibility

Atopic patients suffering from sensitization to *Lol p* were selected from among the subjects of an urban population of Buenos Aires, Argentina. Selected subjects had a clinical history of allergic rhinitis and/or asthma with a seasonal pattern of symptoms suggestive of pollinic allergy. Atopy was determined by positive RAST and positive skin prick test responses to ryegrass pollen, according to the manufacturer's instructions but negative against *A. fumigatus* and *P. americana*. Healthy individuals with no history of allergy and with negative responses to pollens and protease-containing allergen extracts were included as non-atopic controls.

Patients' sera

Blood to be used for serum component analysis was allowed to coagulate at 4 °C and centrifuged at 1000 g for 15 min. Non-haemolysed serum was separated, and aliquots were stored at –70 °C until use.

Radio-allergoadsorbent test

Determination of specific IgE in human sera was performed as described previously [17].

Skin testing

Skin tests were carried out to measure reactivity to allergenic test and control extracts according to Aas and Belin [18]. Skin tests were performed in the morning and

graded after 15 min based on the weal size of the histamine control, as it was conventionally established. On the day the skin test was performed, none of the allergic patients or the controls was taking steroids, antihistamines or immunosuppressive drugs. Informed consent was obtained from all subjects before testing. To determine the residual biological activity of mixtures on storage, an *in vivo* intradermal skin testing was performed using mixtures and control extracts before (time 0) and after (90 days) mixture storage. Subjects were skin tested to test and control extracts according to Turkeltaub et al. [19], to select the optimal dilution. Residual biological activity was calculated according to: remaining erythematic diameter after storage $\times$ 100/total erythematic diameter.

Enzyme-linked immunosorbent assay

ELISA assays were performed on mix and single samples as described previously [20], with some modifications. Briefly, a microtitre plate was coated with 50 μ L per well (100 μ g/mL in sodium carbonate buffer pH 9.6) of pollen allergen extract, incubated at 37 °C for 90 min and blocked with 3% defatted milk in 0.1 M PBS. After washing, the wells were incubated with 100 μ L of a 1:10 dilution of human pollen-positive pooled sera overnight at 37 °C. After additional washing with PBS-T, 100 μ L of a 1:2000 dilution of peroxidase-conjugated goat anti-human IgE was added to each well for 2 h at 37 °C. The reaction was developed with *o*-phenyldiamine in citrate buffer, pH 4.6, and hydrogen peroxide. The absorbance was read at 492 nm utilizing an automated ELISA reader (MR 700, Dynatech Lab, Chantilly, VA, USA). Each assay was run in three separately prepared duplicates.

Inhibition enzyme-linked immunosorbent assay

Specific IgE inhibition for *Lol p* was performed in duplicate to determine the loss of potency of pollen allergen extracts in mixes containing protease-rich extracts during storage. ELISA was carried out as mentioned above, but equal parts of glycerinated and non-glycerinated pollen allergen extract mixes (0.1 mg/mL) corresponding to different times of storage or PBS and a 1:10 dilution of human pooled sera containing specific IgE antibodies to that allergen were pre-incubated for 2 h at 37 °C and added to the coated antigen as described above. Sera from non-atopic subjects and protease-rich allergen extracts were used as negative controls for both ELISA and inhibition ELISA assays. The percentage of inhibition in each time of storage was calculated as total reactivity – remaining reactivity after adsorption $\times$ 100/total reactivity.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Ten micrograms of allergen single extract or mixtures were run in 10% polyacrylamide mini-gels in the presence

of sodium dodecyl sulphate, according to Laemmli [21]. Protein bands from *Lol p* pollen extract separated by SDS-PAGE were stained using a modified silver staining [22], for identification using mass spectrometry.

UV-MALDI-TOF-MS analysis

The selected bands were manually excised from the gel and destained with a 50% (v/v) mixture of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate for 20 min at room temperature. Gel pieces were first extensively washed with 50 mM ammonium bicarbonate (pH 8.3) and then acetonitrile, before drying under vacuum. Proteolytic in-gel digestion was performed overnight with sequencing grade trypsin (Promega, Madison, WI, USA) at 35 °C [22]. Peptides were extracted from gels with 60% acetonitrile in 0.2% trifluoroacetic acid, concentrated by vacuum centrifugation and desalted using POROS 10 R2 (Applied Biosystems, Foster City, CA, USA) home-made micro-columns. Peptides were eluted from micro-columns with 3 μ L of matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid in 60% acetonitrile, 0.2% trifluoroacetic acid) directly into the mass spectrometer sample plate. MALDI-TOF peptide mass analysis was performed. MALDI-TOF MS measurements were carried out in a Voyager DE-PRO system (Applied Biosystems) equipped with a N₂ laser source (337 nm). Peptide masses determined in the reflector mode were internally calibrated with autolytic fragments of trypsin. Proteins were identified after database searching with monoisotopic peptide masses using the MASCOT program including the following searching parameters: taxonomy, Eukaryota; monoisotopic mass tolerance 0.05 Da; and partial methionine oxidation.

Western blotting

SDS-PAGE-resolved proteins were electro-transferred onto a nitrocellulose membrane as described by Towbin et al. [23]. Briefly, the blocked membranes were incubated with a 1/10 dilution of *Lol p*-hypersensitive patients's sera in PBS overnight at 4 °C and then incubated with a 1:1000 diluted goat anti-human IgE-peroxidase conjugate for 90 min at room temperature. Sera from non-atopic subjects were taken as negative controls. Colour development was performed with 4-chloro-1-naphthol and hydrogen peroxide in a methanol/PBS solution.

Proteolytic assays

Gelatinase zymography was carried out in polyacrylamide gels as described above, except that gelatine, co-polymerized as a substrate, was added to the solution at a final concentration of 0.15%, according to Duschak et al. [24]. Azocasein digestion assay was performed as described by Duschak et al. [25].

Gel filtration

Extract and mixes were analysed using a gel filtration Superose 12 column on an FPLC analyser. The mobile phase was 10 mM Tris-HCl pH 7.6 with a flow rate of 0.5 mL/min. Peaks were monitored at 280 nm.

Statistical analysis

Differences between samples corresponding to different times of storage for inhibition ELISA were analysed using the Newman-Keuls multiple comparison test. A *t*-test was used for independent samples. Comparisons of the erythematic values produced by samples before and after storage in selected atopic patients were evaluated by Wilcoxon's test (Mann-Whitney *U*-test). Differences were considered to be statistically significant at *P*-values <0.05.

Results

Proteolytic activity of individual extracts used in mixes

The proteolytic activity displayed by the different proteases present in each extract was tested using gelatin containing SDS polyacrylamide gels (Fig. 1A). Zymogram distaining resulted in clear zones where lysis of gelatin had occurred. *Lol p* pollen extract showed a lower amount of gelatinases compared with the pattern obtained by the two non-pollinic analysed extracts. Activity bands located about 97, 66 and 60 kDa of apparent molecular weight could be detected. By contrast, the proteolytic activity found in the *P. americana* and *A. fumigatus* extracts was substantially higher than that was observed in the pollinic extract. *P. americana* showed a complex pattern of gelatinolytic activity bands, whereas *A. fumigatus* showed bands of about 180–200 kDa, a band of 66 kDa and another about 34 kDa, in accordance with previous data [5, 6]. In addition, *P. americana* showed a higher gelatinolytic activity compared with that of the *A. fumigatus*

extract, when loading similar protein amounts. Similarly, non-pollinic extracts also showed higher activities on azocasein compared with the *Lol p* pollen extract (Fig. 1B).

Protein degradation profile during storage of mixes

The degradation of proteins from *Lol p* pollen extracts after storage, with or without the addition of *A. fumigatus* or *P. americana*, was examined by monitoring absorbance at 280 nm in samples submitted to gel filtration in a Sup 12 column using an FPLC system (Fig. 2) and by SDS-PAGE, followed by silver staining. Although the *Lol p* pollen extract did not show significant changes in its protein profile over this period, showing only a low increase of the A_{280} values in the elution range corresponding to proteins of low molecular weight (Figs 2a and d), mixing of pollen allergens with non-related extracts led to drastic changes in protein profiles after storage. *P. americana* led to the disappearance of a peak eluting about 10 mL (Figs 2b and e), and self-proteolysis resultant after storage due to *A. fumigatus* was evidenced by changes in the peak eluting at 20 mL and the increase of A_{280} values eluting at 25 mL, in fractions corresponding to small proteins (Figs 2c and f). In contrast, when mixtures were stored in the presence of glycerol used as a preserving agent, almost no differences were observed in protein profile either in the gel filtration-FPLC analysis or in the SDS-PAGE gels after storage (data not shown).

Analysis of *L. perenne* pollen allergen degradation in mixes containing protease-rich extracts

The monitoring of degradation profile from *Lol p* pollen-specific allergens stored with fungal or cockroach extracts was performed by immunoblotting assays, confronted with human pooled sera. All samples of control *Lol p* pollen taken off within 90 days of storage showed no significant loss of intensity (Fig. 3I, a). Similarly, when the *Lol p* pollen extract was mixed with either *A. fumigatus* or *P. americana* extract, the immune recognition of the *Lol p* allergens located between 45 and 66 kDa by patient pooled sera was almost unaltered during storage, indicating its resistance to proteolysis (Figs 3I, B and C, respectively). By contrast, the addition of each non-related extract to *Lol p* allergen extract resulted in the complete disappearance of the 29-kDa allergen IgE-reactive band. The disappearance was complete within 1 day of storage simultaneous with the appearance of immune-reactive bands lower than 29 kDa in mixes containing *A. fumigatus* (Fig. 3I, B) or after 15 days of storage in the presence of *P. americana* (Fig. 3I, C), allowing the identification of allergens susceptible to proteolytic degradation. In addition, the immune recognition of an allergen of low molecular weight of about 18 kDa also disappeared within 15 days of storage in mixes with *A. fumigatus* or *P. americana* (Figs 3I, B and C, respectively). The

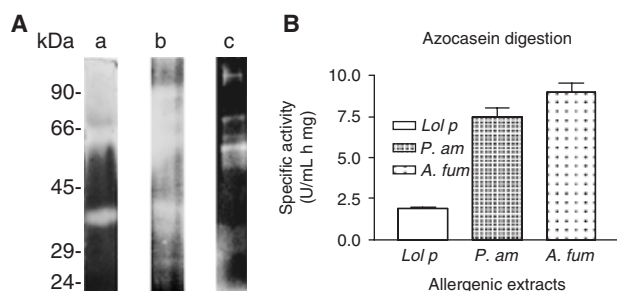


Fig. 1. (A) Zymography of the allergenic extracts. Ten micrograms of *Aspergillus fumigatus* (*A. fum*) (a), *Periplaneta americana* (*P. am*) (b) or *Lolium perenne* (*Lol p*) (c) extract were run on an SDS-PAGE gel containing 0.15% gelatin. Coomassie blue staining led to clear areas representing gelatinase activity. (B) Proteolytic digestion using azocasein as a substrate, expressed as specific activity (units/mL/h/mg) of the different allergenic extracts.

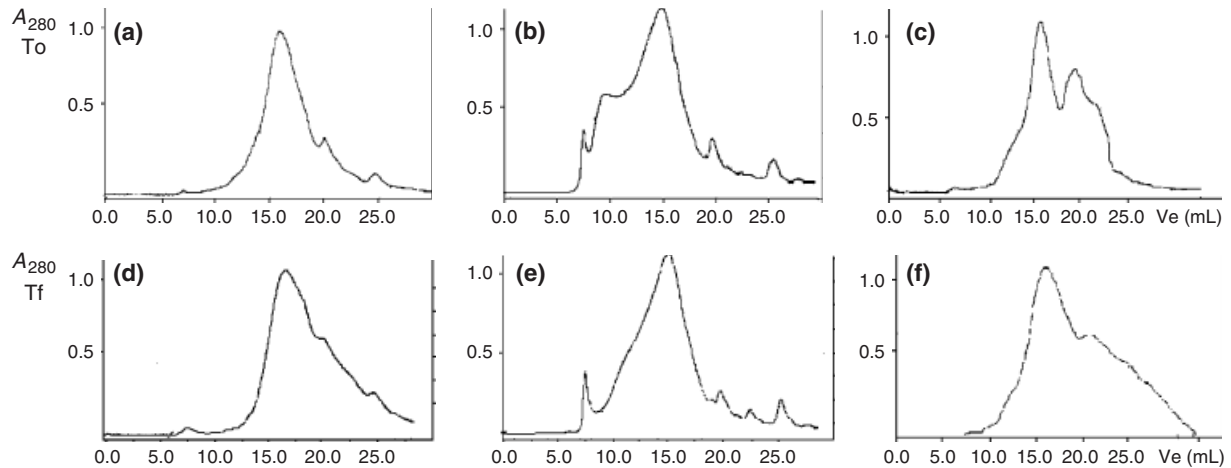


Fig. 2. Sample protein profiles monitored by A_{280} nm before (a, b and c) and after (d, e and f) storage subjected to Sup12 gel filtration column passage corresponding to control *Lolium perenne* (*Lol p*) pollen (a and d) and mixes containing *Lol p* pollen plus *Periplaneta americana* (b and e) or *Aspergillus fumigatus* (c and f) without glycerol as a preserving agent.

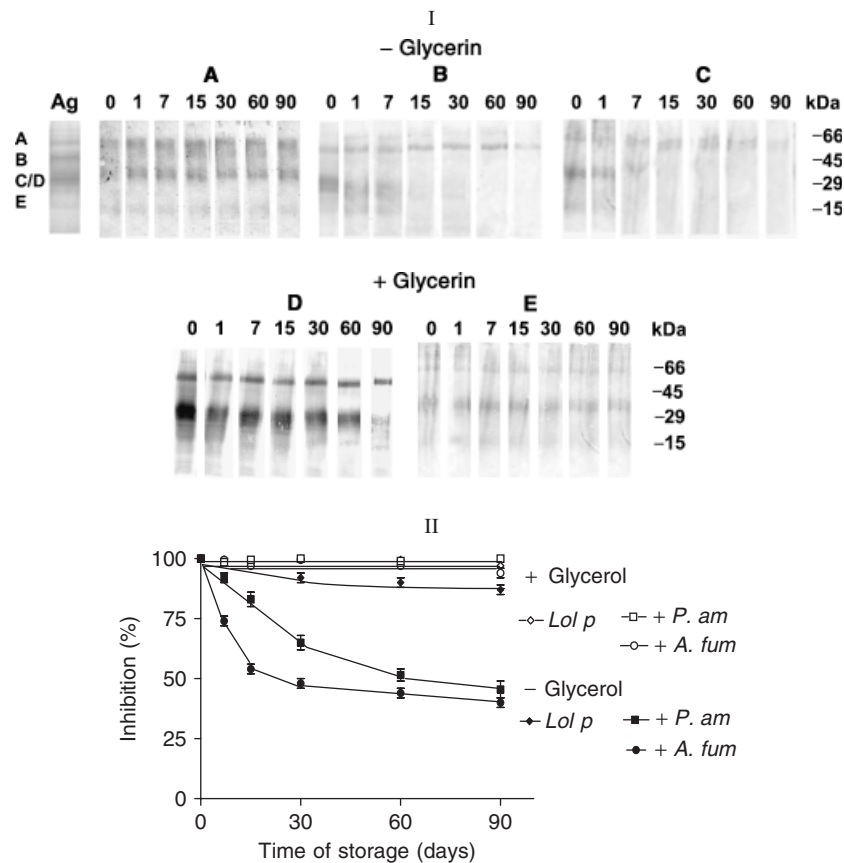


Fig. 3. (I) Immunoblot analysis of single extracts (panel A) (5 μ g protein/lane) and mixtures (10 μ g protein/lane) with (panels D and E) or without glycerol 50% (panels B and C) confronted with atopic patients' antisera. Panels: A, *Lol p*-PBS; B and D, *Lol p*-*A. fum* mixture; C and E, *Lol p*-*P. am* mixture. Numbers on the top of the figures indicate the duration of storage in days. On the left, the lane Ag corresponds to silver-stained SDS-PAGE of *Lolium perenne* extract allergenic bands before mixing with protease-rich extracts. Six protein bands were named from A to E for further identification. (II) IgE ELISA inhibition. Percent inhibition of IgE recognition to *L. perenne* allergen extract by pooled sera of atopic patients to *Lol p* pollen after pre-incubation of a dilution 1 : 10 from human pooled sera from atopic patients with equal amounts of glycerinated and non-glycerinated mixes containing *A. fum* or *P. am* as protease-rich extracts taken off at different times of storage. *A. fum*, *Aspergillus fumigatus*; *Lol p*, *Lolium perenne*; *P. am*, *Periplaneta americana*.

deterioration effect due to mixing *Lol p* with either *A. fumigatus* or *P. americana* extracts was avoided in the presence of glycerol (50%) (Figs 3I, D and E, respectively). These results are in accordance with the inhibition profile of IgE recognition to ryegrass allergens recorded by ELISA inhibition assays (Fig. 3II). When pooled sera from atopic patients were pre-incubated with fungal or cockroach extract samples, as an inhibitor, the percentage of IgE recognition increased abruptly up to 40% on day 15 or decreased gradually along 60 days of storage. The fact that *A. fumigatus* showed a lower inhibition effect on pollen allergens compared with the *P. americana* extract, particularly in the first month of storage, suggests a higher degradation by proteolysis in the presence of the fungal extract. Anyway, degradation was avoided when both mixes were stored in the presence of 50% glycerol (Fig. 3II).

Identification of bands susceptible to degradation by proteomic analysis of pollen extracts

A proteomic approach, enabling rapid identification and analysis of proteins, was used to identify the selected immune-reactive bands, based on susceptibility to degra-

dation during storage in mixes. MALDI-TOF reflector spectra of tryptic peptides from the different selected bands of *L. perenne* extract were obtained. Intense ions corresponding to protonated tryptic peptides of each protein band were detected with mass errors lower than 100 ppm. Database searches using peptide masses from each spectrum indicated that the most likely protein in band A from stored samples (Figs 3I B and C; Table 1) matched to Q5TIW3_LOLPR with a score of 83 corresponding to a fragment of the pollen allergen *Lol p 4* from *L. perenne* (perennial ryegrass), presenting a nominal mass (M_r) 46950 and a calculated pI value of 8.87. The analysis of two bands lower than 29 kDa obtained during storage (Fig. 3I, B; Table 1) allowed to identify the co-migrating bands C and D as the allergen forms *Lol p 1* and *Lol p 5*, respectively. One of them matched to AAA63278 (score 89, M_r 27285 and calculated pI 5.8) and corresponded to *Lol p 1* (allergen R7). The other one matched with a protein presenting in database the accession number gi/4416516 with a MASCOT score of 107, corresponding to a protein with M_r 29749 and a calculated pI of 5.62 identified as a *Lol p 5A* precursor. Finally, E from stored samples (Figs 3I, B and C; Table 1) matched to E985064 (score 74, M_r 16242 and calculated pI 5.69),

Table 1. Identification of *Lolium perenne* pollen allergens by MALDI-TOF MS

Band	Allergen	Tryptic peptides from protein bands and matches to <i>Lol p</i> allergens						Sequence coverage (%)	MASCOT score
A	<i>Lol p 4</i>	1	AVVCGRRYDV	RIRVRSGGHD	YEGLSYRSLO	PENFAVVDLN	QMR AVLVDGK	21	83
		51	<u>ARTAWVDSGA</u>	<u>QLGELYAIS</u>	<u>KYSRTLAFFA</u>	GVCPTIGVGG	NLAGGGFGML		
		101	LRKYGAAEN	VIDVKLVDAN	GKLHDKKSMG	DDHFVAVRGG	GGESFGIVVS		
		151	<u>WQVKLLPVPP</u>	<u>TVTIFKIPKS</u>	<u>VSEGAVDIIN</u>	<u>KWQLVAPQLP</u>	<u>ADLMIRIIAM</u>		
		201	GPKATFEAMY	LGTCCTLTPM	MQSKFPELGM	NASHCNEMSW	IESIPFVHLG		
		251	HRDSLEGDLL	NRNNTFKPFA	EYKSDYVYEP	FPKSVWEQIF	GTWLVKPGAG		
		301	IMIFDPYGAT	ISATPEAATP	FPHRKGVLFN	IQYVNYWFAP	GAGAAPLSWS		
		351	KEIYNMEPY	VSKNPRQAYA	NYRDIDLGRN	EVVNGVSTYS	SGKVGWQKYF		
		401	KGNFERLAIT	KGKVDPTDYF	RNE				
C	<i>Lol p 5</i> (precursor)	1	MAVQKYTVAL	FLAVALVAGP	AASYAADAGY	TPAAAATPAT	PAATPAAAGG	39	107
		51	<u>KATTDEQKLL</u>	<u>EDVNAGFKAA</u>	<u>VAAAAANAPPA</u>	<u>DKFKIFEAAF</u>	SESSKGLLAT		
		101	<u>SAAKAPGLIP</u>	<u>KLDTAYDVAY</u>	<u>KAAEGATPEA</u>	<u>KYDAFVTALT</u>	<u>EALRVIAGAL</u>		
		151	<u>EVHAVKPATE</u>	<u>EVPAAKIPTG</u>	ELQIVDKIDA	AFKIAATAAN	AAPTNDKFTV		
		201	FESAFNKALN	ECTGGAYETY	<u>KFIPSLEAAV</u>	<u>KQAYAATVAA</u>	<u>APEVKYAVFE</u>		
		251	<u>AALTKAITAM</u>	TQAQKAGKPA	AAAATGAATV	ATGAATAAAG	AATAAAGGYK		
		301	A						
D	<i>Lol p 1</i>	1	ALFAVFLGSA	HGIKVPVPGP	NITAEYGDKW	LDASTWYGK	PTGAGPKDNG	25	89
		51	GACGYKDVDK	<u>APFNGMTGCG</u>	<u>NTPIFKDGRG</u>	CGSCFEIKCT	KPESCSGEAV		
		101	TVTITDDNEE	PIAPYHFDLS	GHAFGSMACK	GEEQNVRSG	<u>ELELQFRRVK</u>		
		151	CKYPDDTKPT	FHVEKGSNP	<u>YLAILVKYVD</u>	GDGDVVAVDI	KEKGDKWIE		
		201	<u>LKESWGAVWR</u>	<u>IDTPDKLTGP</u>	<u>FTVRYTTEGG</u>	TKSEVEDVIP	EGWKADTSYS		
E	<i>Lol p 1A</i>	251	AK					22	74
		1	NEEPIAPYHF	DLSGHAFGSM	AKKGEEQKLR	<u>SAGELELQFR</u>	RVKCKYPDGT		
		51	KPTFHVEKGS	NPNYLAILVK	YVDGDGDVVA	VDIKEKGKDK	<u>WIELKESWGA</u>		
		101	<u>VWRIDTPDKL</u>	<u>TGPTVRYTT</u>	EGGTKSEVED	VIPEGWKADT	SYSAK		

Database searching results from peptide mass fingerprints obtained from MALDI-TOF spectra. Matched peptides are underlined bold.

Lol p, *Lolium perenne*.

which was identified as a partial *Lol p 1A* allergen from perennial ryegrass.

Loss of biological potency as measured by the residual biological activity of extract mixes by *in vivo* intradermal skin test assay

To determine the effect of fungal and cockroach high protease-containing extracts on the biological activity of allergen ryegrass extract, an *in vivo* intradermal skin test assay was performed using a single *Lol p* pollen extract and mixes as allergenic samples in atopic-selected patients. Pollinic extract co-incubated separately with both non-related extracts lost a statistically significant amount of biological activity, as measured by diminution of erythematic diameter compared with saline co-incubated controls. As expected, when mixtures contained *A. fumigatus* extract, the loss was higher than those containing *P. americana* allergens, reinforcing the previous results. Ryegrass pollen retained almost the entire original allergenic potency after storage. Conversely, ryegrass pollen extract retained approximately 50% and 60% of its biological activity when it was stored for 90 days as a mixture with the fungal or the cockroach extract, respectively. When glycerol was added to both mixtures, the loss of biological activity was significantly minimized (Fig. 4).

Discussion

It is well known that the stability of allergen extracts may be altered by several factors, including proteolytic effects

[2–4, 7, 27–29]. To avoid them, it is generally recommended not only to mix different allergen extracts for hyposensitization therapy [3] but also to inject them into different arms. However, mixing of multiple cross-reactive or unrelated allergens from various source materials to be used for desensitization protocols is a common practice, although this procedure can lead to altered immunologic activity of some compounds, thus affecting the quality of vaccines used for immunotherapy of allergic disorders. We have used SDS-PAGE and MALDI-TOF MS methods as suitable tools to identify specific allergens susceptible to degradation by proteolysis in mixes with extracts from either *P. americana* or *A. fumigatus*, containing high proteolytic activity [5, 6] when stored as a mixture without glycerin. The use of MALDI-TOF MS to identify specific degradation products due to this well-known stability phenomenon offers for the first time potential resolving power not possible with the use of other techniques. In addition, the residual allergen biological activity retained after the proteolytic degradation of specific pollinic allergens was calculated, reinforcing the clinical relevance of this phenomenon. In addition, taking into account that the stabilizing properties of glycerol on proteins have been well documented for allergen extracts [2, 3, 30, 31] and that the protective effects of glycerinated commercially available protease-rich allergen extracts were also described [32], the stability of the immunologic activity of extract components in protease-rich mixes was avoided using glycerol as a protective agent.

It is worth noting that the low proteolytic activity detected in the pollen extract by either gelatinase zymography or degradation of azocasein is in accordance with the fact that single pollinic extracts are considered to be very stable. Besides, protein profiles of stored protease-rich mixes compared with those recorded by fresh mixes showed differences confirming protein degradation. In addition, the degradation of specific major allergens when pollen allergens are mixed with non-pollinic extracts correlated both with the decrease in relative potency as measured by the ELISA inhibition test and the diminution in residual biological activity evidenced by an intradermal skin test. Finally, using an intradermal skin assay as a biological *in vivo* test system, a significant decrease of the allergenic potency of allergen pollens was observed in both mixtures after 90 days of storage (Fig. 4), confirming that this procedure leads to altered immunologic activity of some allergen compounds, thus affecting the quality of vaccines used for immunotherapy of allergic disorders when mixes are prepared without stabilizing agents.

Not all the pollinic allergens in extract mixtures are equally susceptible to the activity of proteinases. Thus, the fact that the efficiency of extracts used for diagnostic and therapeutic purposes depends not only on the individual sensitization of the patients but also on the level and stability of relevant allergens in the pollen extract used

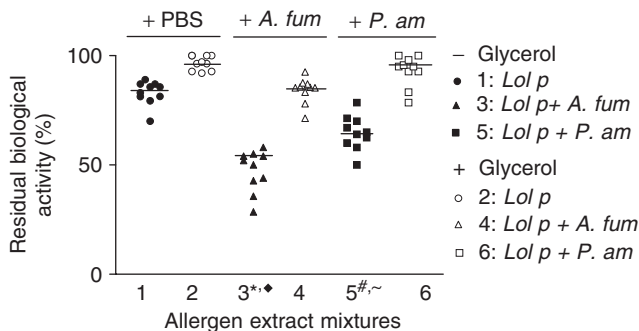


Fig. 4. Residual biological activity of pollinic mixtures on storage. Experimental mixtures of *Lolium perenne* (*Lol p*) allergen extracts with *Aspergillus fumigatus* (*A. fum*) or *Periplaneta americana* (*P. am*) were compared with single pollen extracts mixed with PBS in the presence or in the absence of glycerol. The residual biological activity of pollinic mixtures after 90 days of storage was calculated in atopic patients by *in vivo* intradermal skin testing according to: remaining erythema diameter after storage $\times$ 100/total erythema diameter. Each point represents the mean percentage of the residual biological activity of experimental samples for each patient. * $P < 0.01$, 3 (*Lol p* + *A. fum*) vs. 1 (*Lol p* + PBS); $\diamond P < 0.05$, 3 (*Lol p* + *A. fum*) vs. 4 (Glycerinated *Lol p* + *A. fum*); $\#P < 0.05$, 5 (*Lol p* + *P. am*) vs. 1 (*Lol p* + PBS); $\sim P < 0.05$, 5 (*Lol p* + *P. am*) vs. 6 (Glycerinated *Lol p* + *P. am*).

signifies an urgent need to identify particularly the major antigenic determinants susceptible to degradation by proteases, to prevent this negative aspect for desensibilization treatments. In this regard, although identification of fragments that occur after the proteolytic processing was difficult due to the presence of other proteins in mixes containing either *A. fumigatus* or *P. americana* added to the sample, the combination of SDS-PAGE, western blot and MALDI-TOF analysis provided suitable tools for identification of specific pollen allergens susceptible to proteolysis in mixes. Interestingly, a low-molecular-weight band and additional fragments were detectable, resulting in fragments of allergens that naturally exist in the original extract. Unfortunately, we failed to identify band B. However, the band A located between the markers of 45 and 66 kDa, which showed immune-reactivity with patient sera and was resistant to degradation by proteases with storage in the mixes described here, was further identified as a fragment of *Lol p 4*. In accordance with our findings, group 4 allergens were described as basic glycoproteins of 50–60 kDa molecular weights with a pI about 9.4 in different grass species [33], and an allergen from group 4 in *Phleum pratense* was characterized as a trypsin-resistant protein [34]. In contrast, other bands underwent degradation during storage. Among them, particularly those located in the range 25–35 kDa immune-recognized by atopic patient sera were subjected to mass spectrometry analysis. Band C was identified as a form of *Lol p 1* allergens from perennial ryegrass, whereas D was identified with the highest MASCOT score as an *Lol p 5A* precursor. Similarly, allergens from other pollen species such as *Phl p 1* and *Bet v 1*, when stored in mixtures with the mould *Fusarium culmorum*, underwent some degradation whereas *Phl p 5* and *Bet v 6* were almost entirely degraded early during storage with mould-rich protease [4]. Accordingly, group I allergens from grass pollen are plant glycoproteins of about 30 kDa [35]. The literature has shown some contrasting data related to proteinase activity in *Lol p 1* depending on homology to the C1 family of cysteine proteinases or lack of protease activity [36, 37]. Although we did not observe gelatinolytic activity in *Lol p* pollen about 30 kDa under the condition tested, the fact that this *Lol p 1* displayed other proteolytic activities should not be ignored.

In conclusion, our findings showed that proteases from different sources, fungal and American cockroach species, when co-incubated in the same vial with ryegrass pollen in the absence of glycerol (50%) as a stabilizing agent clearly affected the overall pollinic biological activity by degradation of specific major pollen allergens within a short period of time. The use of the latest tools in proteomics sheds a new light on the study of allergen standardization, allowing, for the first time, to identify *Lol p 1* and *Lol p 5* as the relevant allergens responsible for the loss of pollinic potency in the mixtures.

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