

Quantification in mass units of group 1 grass allergens by a monoclonal antibody-based sandwich ELISA

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Summary

Background Grass pollen extracts currently used for allergy diagnosis and immunotherapy are a complex mixture of proteins of which only a few have allergenic activity. Lol p 1 is one of the most important allergens in grass pollen extracts.

Objectives To develop a two-site enzyme-linked immunosorbent assay for the quantification of Lol p 1 and other group 1 allergens from grass species, and to assess its suitability for quantifying this group of allergens.

Methods Balb/c mice immunized with recombinant Lol p 1 were used for the production of monoclonal antibodies. Screening of hybridomas was performed by direct ELISA, and selected monoclonal antibodies were immobilized on ELISA plates and incubated with samples containing group 1 allergens. Bound allergens were detected by a combination of biotinylated Lol p 1-specific monoclonal antibody and peroxidase-streptavidin conjugate.

Results The assay is based on three Lol p 1-specific monoclonal antibodies with different epitope specificities. The optimized ELISA measured Lol p 1 concentrations ranging from 125 to 1000 ng/mL and could quantify group 1 allergen from grass species belonging to the Pooideae subfamily. The assay does not depend on anti-sera production or availability of human sera and thus reactives can be produced in unlimited amounts.

Conclusion This sensitive and specific Lol p 1 assay will be helpful both for quantifying the group 1 allergen content of Pooideae pollen extracts intended for clinical use and for studying cross-reactivities among pollen extracts.

Keywords: monoclonal antibody, pollen allergy, pollen extract, sandwich ELISA, recombinant allergen, Lol p 1.

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Introduction

Allergies are frequently diagnosed and treated by immunotherapy with whole allergen extracts. These crude extracts of naturally occurring allergenic materials contain complex mixtures of proteins of which only a few have allergenic activity. Due to this biological origin, allergen extracts are also variable in composition, depending on the exact origin of the source of material and the procedure of preparation of extracts [1–3]. Thus, accurate standardiza-

tion of allergen extracts is essential to guarantee their quality. Current standardization methods are based on *in vivo* testing of in-house reference (IHR) extracts in allergic volunteers by intradermal or subcutaneous skin tests. Subsequently, production batches are compared to this IHR using *in vitro* IgE-based methods (e.g. competitive RAST, competitive ELISA) using pooled sera from allergic patients.

This current standardization protocol faces two main problems. First, all these techniques involve the use of human sera which are difficult to standardize. Second, only an overall allergenic potency is determined on the basis of reactivity with human IgE antibodies of pooled sera from

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allergic patients, and extracts with similar potency can have differences in major allergen ratios [4]. As monoclonal antibodies (mAbs) against most major allergens are now available, one way to characterize allergenic extracts is to quantify allergen levels in mass units (μg protein/mL) by specific immunoassays with mAbs. Allergen-specific mAbs have some advantages as they show specificity for a single epitope and can be produced in unlimited amounts.

Eleven allergen groups have been described up to now in grass pollen but only eight of them represent major allergens [5]. Groups 1 and 5 allergens are the most predominant with an IgE prevalence $> 80\%$ [5,6]. Methods for specific quantification of grass allergens are already available [7–10] but none of them are for the major allergens from group 1.

In this study, a solid phase ELISA based on Lol p 1-specific mAbs was developed for the first time in order to provide a specific and sensitive estimation of the content of this major allergen in grass pollen extracts. The method is based on the use of three different mAbs recognizing non-overlapping epitopes of Lol p 1.

Material and methods

Amplification of the gene encoding Lol p 1 by PCR

Poly(A⁺)-enriched RNA was isolated from *Lolium perenne* pollen (Mediterráneo Polen, Jaén, Spain) using the Quick Prep MicroRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). First strand cDNA was synthesized from 1 μg poly(A⁺)-enriched RNA by using a First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech). The coding sequence for the Lol p 1 protein was amplified using polymerase chain reaction (PCR) with the following primers (Sigma-Genosys, Cambridge, UK), which were derived from the previously reported nucleotide sequence [11]: LOLF, CCGGATCCACATATGATCGGAAGGTACCACC and LOLR, CCAAGCTTTCAC TTGGCCGAGTAGG. *Bam*HI, *Nde*I, and *Hind*III restriction sites, respectively, are underlined. A DNA fragment was isolated by GeneClean (Bio101, La Jolla, CA, USA) and cloned into pBluescript II SK (Stratagene, La Jolla, CA, USA). Restriction analysis and recombinant DNA techniques were performed by standard methods [12] and nucleotide sequence was determined by the dideoxy chain termination method [13] using an ABI 373 A DNA sequencer (Applied Biosystem, Foster City, CA, USA).

Expression and purification of rLol p 1

The complete gene encoding Lol p 1 was digested with *Bam*HI and *Hind*III and subcloned into the pTrc-HisB

vector (Invitrogen, Groningen, the Netherlands). After transformation of *E. coli* DH5 α and analysis, recombinant cells were grown in Luria-Bectani medium (LB) with 100 $\mu\text{g}/\text{mL}$ ampicillin. When cultures reached an OD₆₀₀ of 0.6, 0.6 mM isopropyl-thio- β -galactoside (IPTG) was added to the culture, and incubation continued for another 3 h. Cells were harvested by centrifugation, and the pellet, resuspended in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5 was lysed by lysozyme treatment (100 $\mu\text{g}/\text{mL}$, 15 min, 37 °C) and mild sonication. rLol p1 was purified by the TALON metal affinity resin (Clontech, Palo Alto, CA, USA) following the manufacturer's recommendations.

Allergen extracts and purified natural allergens

Allergen extracts were obtained from Bial-Aristegui (Bilbao, Spain). nLol p 1 was purified from *Lolium perenne* pollen extract by a combination of ion exchange chromatography and gel filtration. Briefly, a crude extract was applied to a High Load Sepharose Q 16/20 column equilibrated with 20 mM Tris-HCl pH 8.0 in a ÄKTA-prime™ system (Amersham Pharmacia Biotech), and nLol p 1 was eluted with a 0–400 mM NaCl gradient. Lol p 1-containing fractions were pooled, concentrated and applied to a Superdex S-200 16/60 column equilibrated with 20 mM Tris-HCl pH 8.0 plus 150 mM NaCl. Finally, Lol p 1-containing fractions were pooled and applied to a monoQ column in a SMART™ system (Amersham Pharmacia Biotech) under the conditions described above.

Production of mAbs

Balb/c mice were immunized intraperitoneally with 100 μg of rLol p1 emulsified in complete Freund's adjuvant. The same amount of antigen with incomplete Freund's adjuvant was given every 2 weeks for 2 months. Anti-Lol p 1 reactivity of mouse sera was checked by ELISA using 200 ng of purified rLol p 1 and nLol p 1 to coat ELISA wells. A final 100 μg intraperitoneal dose without adjuvant was administered 3 days before splenectomy. Production of mAbs was performed as described by Köhler and Milstein [14]. Isotype detection was done by IsoStrip kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Purification and biotinylation of antibodies

mAbs were purified from hybridoma culture supernatants with a HiTrap Protein G (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Purified IgG was labeled with biotin using the ECL™ protein biotinylation module (Amersham Pharmacia Biotech). Briefly, purified antibody was diluted in 1 mL of 40 mM

bicarbonate buffer, pH 8.6 at a concentration of 1 mg/mL, and incubated with 40 µL of biotinylation reagent for 1 h at room temperature. The sample was run into a Sephadex G-25 column equilibrated with PBS-BSA 1%, and eluted with PBS. UV absorbance at 280 nm of collected fractions was measured in order to detect the fractions containing the eluted protein.

ELISA

For screening of anti-Lol p 1 reactivity in mice serum and hibridomas, microtitre plates (Maxisorp, NUNC, Denmark) were coated with 2 µg/mL of Lol p 1 in 0.1 M bicarbonate buffer pH 9.6, and blocked with 1% BSA, 0.05% Tween 20 in PBS (PBS-B-T). Afterwards, hibridomas supernatants were added and incubated for 1 h at 37 °C, and detection carried out as previously described [15].

For ELISA-inhibition experiments, different concentrations of competitor purified mAbs were added to the wells and incubated for 1 h, and then purified labelled mAbs 9A4, 7A8 or 9F6 were added and developed as previously described [15].

Immunoblotting

Proteins were separated by SDS-PAGE according to Laemmli [16] and electrophoretically transferred to polyvinylene difluoride (PVDF, Immobilon-P-membranes, Millipore Corp., Bedford, MA, USA) by the method of Towbin *et al.* [17]. After blocking, the blot was incubated for 1 h at 37 °C with mAbs, washed and incubated in the same conditions with anti-mouse polyvalent immunoglobulins HRP-conjugated (Sigma Chemical Co., St Louis, MO, USA) (1 : 1000 diluted), and developed with 4-chloro-1-naphthol solution.

Quantification of Lol p 1 by mAb-based sandwich ELISA

Microtitre plates were coated overnight at 4 °C with 100 µL of anti-Lol p 1 mAbs 7A8 and 9F6, at 5 and 10 µg/mL, respectively, in 100 mM bicarbonate buffer pH 9.6. After blocking with PBS-B-T for 1 h at 37 °C, 100 µL of serially diluted Lol p 1 (2 µg/mL) or extracts (20 µg/mL) in PBS-B-T were added and incubated for 1 h at 37 °C. After washing, 100 µL of biotinylated mAb 9A4 (1.25 µg/mL in PBS-B-T) was added to each well and incubated for 1 h at 37 °C. Wells were washed three times with PBS-T and the bound complexes were detected by the incubation for 1 h at 37 °C with streptavidin-horseradish peroxidase conjugate (Sigma) at 250 ng/mL in PBS-B-T. After three washes, the peroxidase activity was measured, adding 200 µL/well of a commercial solution of *o*-phenylenediamine (Sigma). After 30 min, colour reaction

was stopped by adding 50 µL/well of 3 M H₂SO₄, and the optical density was read at 492 nm. A standard curve was generated with serial two-fold dilutions (0.125–1 µg/mL) of purified Lol p 1. Crude extracts were assayed as dilution series in duplicate. The concentrations of the sample were interpolated from the linear portion of the standard curve.

Evaluation of assay variation

The detection limit was calculated as the amount of Lol p 1 which corresponded to the mean plus 2.33 times the standard deviation obtained after 30 measurements of the zero standard. In order to evaluate intra-assay variation, 20 microtitre wells were incubated with the same concentration of Lol p 1, with Lol p 1 concentrations ranging from 15.6 to 2000 ng/mL, and the coefficient of variation (CV) was calculated. CV was defined as the standard deviation divided by the mean and multiplied by 100. CVs smaller than 20% were considered not to be significant.

EAST-inhibition

The allergenic activity of the grass extracts was measured by enzyme allergosorbent test (EAST)-inhibition (Hytec-specific IgE EIA; Hycor Biomedical Inc., Kassel, Germany) as described by the manufacturer using paper disks coupled to IHR *L. perenne* extract as solid phase. The allergenicity of grass extracts was calculated by comparison with the corresponding IHR *L. perenne* extract previously standardized by the skin prick test.

Results

Expression and purification of Lol p 1

cDNA coding for Lol p 1 was obtained by reverse transcriptase-PCR from mRNA of *L. perenne* pollen with primers flanking the mature protein designed from the nucleotide sequences reported previously [11,18]. The amino acid sequence of the encoded protein was identical to that reported [11], except for the replacements Asn¹⁴⁶→Lys and Val¹⁴⁷→Leu (numbering of the precursor protein), which resulted in C→G and G→C substitutions in the nucleotide sequence, respectively. Both substitutions have already been reported [18], and demonstrate that there are multiple Lol p 1 isoforms. The insertion of the cDNA encoded Lol p 1 in frame with the His-tag sequence of TrcB-His allows the expression and purification of the fusion protein by affinity chromatography using an Ni²⁺-coupled agarose column. Figure 1 shows SDS-PAGE analysis of expression experiments. Analysis of the gel indicated that the recombinant protein constituted 10–15% of the total bacterial proteins. Recombinant Lol p 1 (rLol p

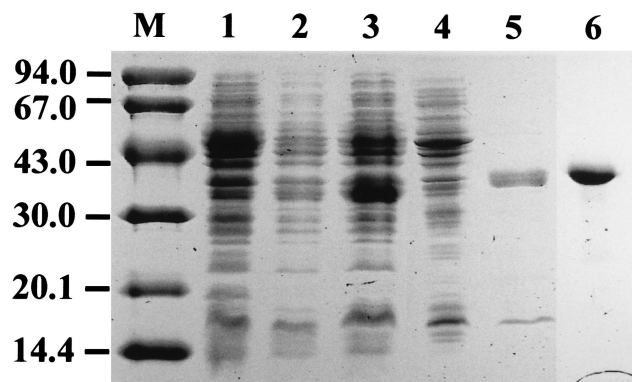


Fig. 1. Coomassie stained SDS-PAGE of extracts from *E. coli* DH5 α containing expression vector pTrc-HisB alone (lane 1), recombinant pTrc-HisB with cDNA encoding Lol p 1 uninduced and induced (lanes 2 and 3), soluble and insoluble protein (lanes 4 and 5) and purified rLol p 1 (lane 6).

1) mainly remained in the insoluble fraction and thus denaturing conditions with 8 M urea were used for purification. Refolding of the denatured protein was achieved by slow dialysis using buffers with decreasing concentrations of urea. The final yield of purified rLol p 1 was 8–9 mg/L cell culture with a purity of 95% as estimated from SDS-PAGE.

Production and characterization of mAbs

Anti-Lol p 1 hybridoma production through immunization with purified rLol p 1 resulted in 12 mAbs with specificity for rLol p 1 and nLol p 1 by ELISA. Based on their reactivity, three mAbs, typed as IgG1 subclass with κ light chains, were chosen for further studies. Reactivity to group 1 allergens of grass pollens was checked by immunoblot with whole pollen extract from grasses of the Pooideae and Panicoideae subfamilies (Fig. 2). The 3 mAbs only recognized the group 1 grass allergens from the Pooideae grass subfamily, but none of them bound to the *Cynodon dactylon* extract from the Panicoideae subfamily. The 3 mAbs reacted with a protein band of 34 kDa and mAb 7A8 reacted also with a smaller band of 30 kDa in extracts of *Phleum pratense*, *Poa pratensis* and *Dactylis glomerata*. Two mAb-reacting bands corresponding to Phl p 1, Poa p 1 and Dac g 1 have previously been described in these extracts [19–21].

Cross-inhibition studies were undertaken using unlabeled mAb in order to inhibit the binding of biotinylated mAb to the allergen (Fig. 3). As expected binding inhibition was observed with the same unlabeled mAb (> 75% when using mAb inhibitor concentrations of 2–4 μ g/mL). In contrast, combination with unrelated unlabeled mAb showed less than 22% inhibition. Therefore, these three

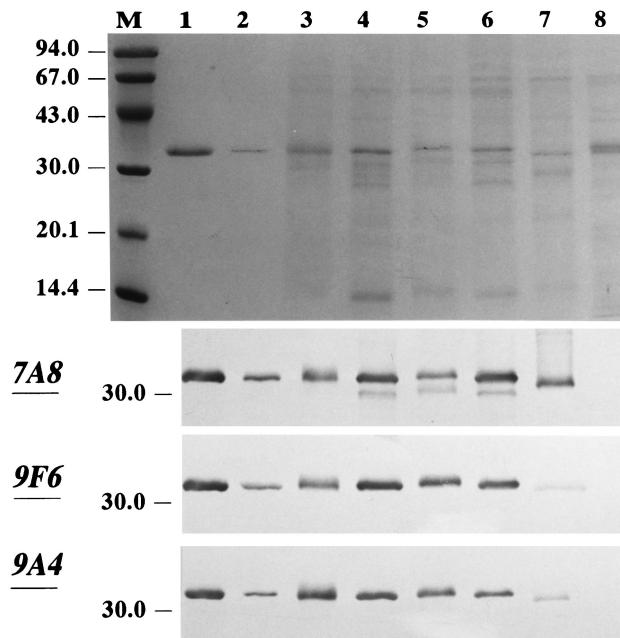


Fig. 2. Coomassie stained SDS-PAGE and Western blot of different proteins and pollen extracts incubated with mAbs. nLol p 1 (lane 1), rLol p 1 (lane 2), pollen extracts of *L. perenne* (lane 3), *P. pratense* (lane 4), *P. pratensis* (lane 5), *D. glomerata* (lane 6), *Secale cereale* (lane 7) and *C. dactylon* (lane 8).

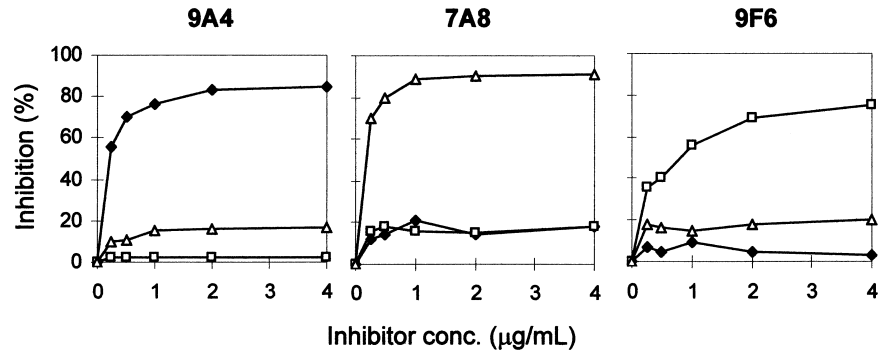
mAbs showing the highest absorbance values against most common grass extracts (Table 1) and specific for the Pooideae subfamily were used for the development of a sandwich ELISA.

Lol p 1 sandwich ELISA

All possible pairs of mAbs were tested for applicability in a sandwich ELISA. The combination of mAbs 7A8 or 9F6 with 9A4 as detector rendered the highest optical densities. Given that the use of more than one mAb is advisable, a mixture of 7A8 and 9F6 was selected as coating mAbs, whilst biotinylated 9A4 was chosen as detector. Figure 4 shows the binding curve of nLol p 1. Using this preparation it was possible to quantify the Lol p 1 content in the range of 125–1000 ng/mL (range of linearity), although the coefficient of interassay variation was less than 20% ($n = 20$) in the range of 125–4000 ng/mL. The sensitivity or detection limit was 31.25 ng/mL. Interference of the skin prick diagnostic solution components (glycerol and phenol) on the Lol p 1 ELISA was tested. No effect of these components was detected at 0.2% of glycerol and 0.002% of phenol.

The Lol p 1 sandwich ELISA was also used to determine the group 1 allergen content in pollen extracts from other grasses (Fig. 4). The amounts varied between 0.25 μ g/mL

Fig. 3. Competitive inhibition of biotinylated mAb to solid-phase Lol p 1 by unlabeled mAb. Each biotinylated mAb was added alone (no inhibition) or in presence of identical or unrelated unlabeled mAb to Lol p 1 coated plates: mAb 9A4 (◆), mAb 7A8 (Δ), and mAb 9F6 (□).



(*P. pratensis*) and 1.05 μg/mL (*H. lanatus*), which constituted a group 1 allergens content relative to the total protein of 5 and 20%, respectively. Nevertheless, the corresponding purified group 1 allergen for each extract should be used as the standard in the dose–response curve. The specificity of the ELISA was ascertained by testing different allergenic plant extracts (Table 2). Different group 1 allergens content was found in the Festuciformes group of the Pooideae grasses, but was negligible in extracts from the Phragmitiformes group of the same subfamily. The group 1 content was also negligible in extracts from weeds (*Plantago*, *Rumex*, *Helianthus* and *Parietaria*), trees (olive tree) and members of the Panicoideae subfamily.

The Lol p 1 content of several production batches of *L. perenne* pollen extract, determined by this specific ELISA, was compared with their allergenic potency, as determined by EAST-inhibition (Fig. 5). A good correlation was obtained between these two assays ($r^2 = 0.85$), and an average of Lol p 1 content of 13 μg/mL in a *L. perenne* extract with an activity of 10 000 UBE/mL could be estimated.

Discussion

Crude allergen extracts are currently used for allergy diagnosis and immunotherapy although their allergen

composition and allergenic potency may vary. This is likely to be due to the variability of the natural source materials, which in turn may depend on the environmental conditions in the case of pollens, the phase of maturation and strain used in the case of moulds, the culture parameters of mites or the type of epidermal material of pets.

In this study, a solid phase ELISA method was set up to measure group 1 Pooideae grass pollen allergens with mAbs. This powerful tool has been applied to the characterization of extracts from allergenically important pollen [9,22–24] and indoor [25–27] allergens. Sandwich immunoassays present several advantages over direct or inhibition ELISA derived from the fact that non-relevant proteins do not interfere with target detection, and that the antigens are detected as native proteins in the liquid phase, thus avoiding any conformational changes after adsorption to the solid phase.

Given that several isoforms of Lol p 1 have been described [11,18,28], the use of more than one mAb is advisable so as to circumvent a potential pitfall of the technique. Consequently, the immunoassay described here is based on the use of two capture mAbs, which recognize non-overlapping epitopes. Moreover, the assay is highly specific for group 1 allergens from the Festuciformes grasses, a group of the subfamily Pooideae that constitute

Table 1. Direct ELISA of different mAbs anti-group 1 allergen to grass extracts

Extract	mAb									Control
	7A8	9F6	9A4	1B5	1B10	4F1	8C12	9A8	9F8	
<i>Lolium perenne</i>	2.926	2.213	2.792	2.750	1.030	2.487	1.409	2.236	2.792	0.165
<i>Phleum pratense</i>	1.524	0.641	1.239	0.308	0.156	0.719	0.173	0.447	0.875	0.144
<i>Poa pratensis</i>	2.805	2.198	2.624	0.829	0.156	2.437	0.165	0.884	2.537	0.170
<i>Dactylis glomerata</i>	2.829	2.377	2.377	2.683	0.129	2.528	0.143	1.986	2.713	0.151
<i>Secale cereale</i>	2.918	0.300	2.868	2.918	0.123	0.147	0.137	2.152	2.965	0.130
<i>Cynodon dactylon</i>	0.141	0.139	0.139	0.124	0.122	0.151	0.148	0.133	0.143	0.143

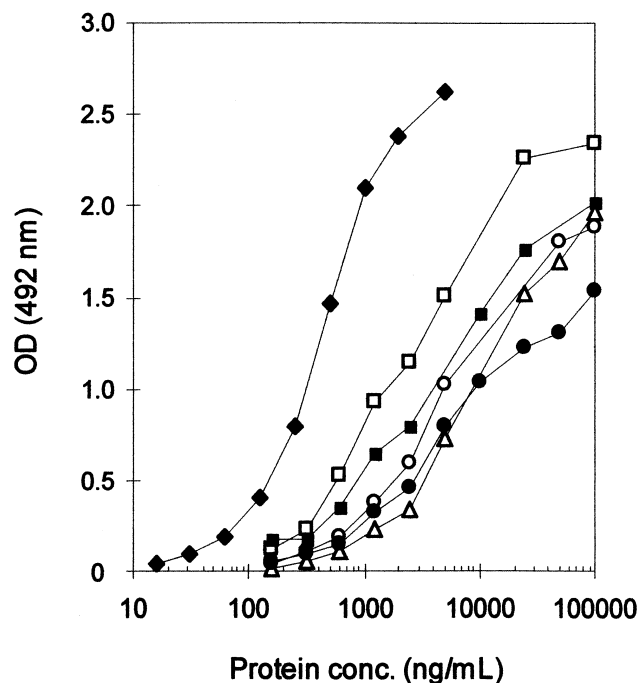


Fig. 4. Dose-response curves in the group 1 allergen ELISA of (◆) purified Lol p 1 and crude extracts of: *L. perenne* (□), *P. pratense* (■), *D. glomerata* (○), *P. pratensis* (Δ) and *S. cereale* (●).

the most important group of grasses involved in seasonal allergic diseases [6]. Amino acid sequences identity among these allergens (Lol p 1, Phl p 1, Hol l 1, Poa p 1 and Pha a 1) are greater than 90%, while Lol p 1 and group 1 allergens from non-Festuciformes grasses (*C. dactylon* and *O. sativa*) share less than 66% identical amino acids. The sensitivity threshold appeared to be 31.25 ng/mL of Lol p 1, comparable in magnitude to the Phl p 4 specific assay [9]. The assessment of day-to-day, inter- and intra-assay variability show that this ELISA is highly reproducible in the linear portion of the standard curve, i.e. over the 0.125–4 µg/mL range. The dose-response curve for *L. perenne* extract was parallel to that obtained with Lol p 1, indicating that the allergen had not been altered during the purification process. As prick test components diluted 250 times from the original solution had no effect on the assay and the sensitivity of the procedure allows dilutions of 1 : 500, this ELISA could be applicable for the routine measurement of group 1 allergen content in production batches of skin prick test diagnostic solutions.

The trend in allergen standardization is moving towards the measurement of major allergens [4,29,30], but the assessment of the total allergenic potency of an extract should not only rely on the quantification of a single allergen [7,31,32]. Recent studies have demonstrated that one–three allergens are necessary for *in vitro* diagnostics and standardization [6,33–35]. Diagnosis for grass pollen allergy can be simplified by replacing grass mixtures with a single species, and even with some purified allergens. Purified group 1 and 5 allergens cover together greater than

Table 2. Specificity of sandwich ELISA for group 1 allergen from plant extracts

	Sample	Protein concentration (µg/mL)	OD _{sample} -OD _{blank} (492 nm)	Group 1 content (µg/mg)
Festuciformes group	<i>Lolium perenne</i>	1.25	0.822	182
	<i>Holcus lanatus</i>	1.25	0.938	209
	<i>Phleum pratense</i>	1.25	0.647	148
	<i>Agrostis alba</i>	1.25	0.599	140
	<i>Trisetum sp.</i>	1.25	0.344	103
	<i>Dactylis glomerata</i>	2.50	0.591	69
	<i>Secale cereale</i>	2.50	0.453	59
	<i>Poa pratensis</i>	2.50	0.334	51
Phragmitiformes group	<i>Phragmites communis</i>	20	0.011	< 1
Panicoidea sub-family	<i>Cynodon dactylon</i>	20	0.010	< 1
	<i>Zea mays</i>	20	0.068	< 1
	<i>Rumex acetosella</i>	20	0.010	< 1
Non-grass plants	<i>Plantago lanceolata</i>	20	0.011	< 1
	<i>Parietaria judaica</i>	20	0.029	< 1
	<i>Helianthus annuus</i>	20	0.010	< 1
	<i>Olea europaea</i>	20	0.010	< 1

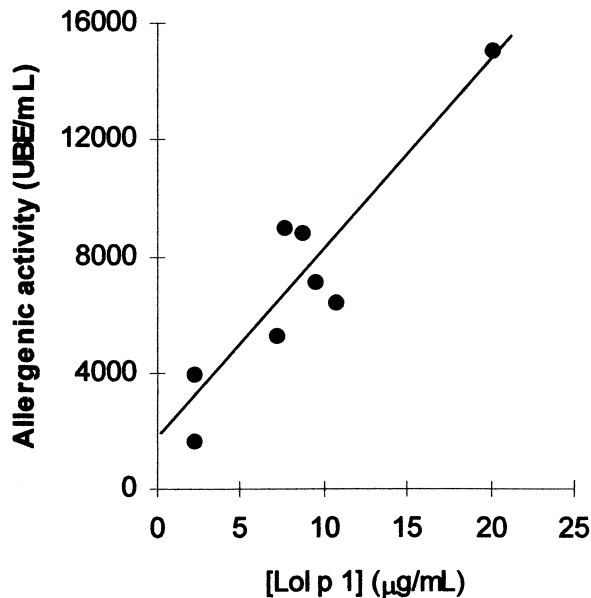


Fig. 5. Correlation between allergenic potency (UBE/mL) determined by EAST-inhibition and Lol p 1 content (µg/mL) measured by the two-site mAb ELISA.

90% of all sera with positive results to grass pollen and approximately 80% of all anti-grass pollen IgE [6]. Three sandwich ELISA for measurement of group 5 allergens have already been developed [7,8,10] that, together with the one described here, could make possible accurate standardization and quality control of grass pollen extracts. Quantification of major allergens is also needed for optimal specific immunotherapy as low-dose immunotherapy is usually ineffective and high doses may induce an unacceptable rate of systematic reactions [36]. There is good evidence for immunotherapy with ragweed, grass, cat, mite and venom allergens that a maintenance dose of 5–20 µg major allergen per injection is associated with significant improvement in patient symptom records [36,37]. The average of Lol p 1 content in maintenance doses is 13 µg, which is in accordance with these data.

In conclusion, this paper describes, for the first time, an ELISA procedure for measurement of group 1 allergens in grass pollen. The assay is highly sensitive to and specific for Pooideae grasses and could be very useful for quality control of preparations of wild and cultivated grasses intended for clinical use.

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