Molecular cloning, expression and immunological characterisation of Lol p 5C, a novel allergen isoform of rye grass pollen demonstrating high IgE reactivity[1]

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Received 19 August 1999; received in revised form 14 October 1999

Edited by Masayuki Miyasaka

Abstract A novel isoform of a major rye grass pollen allergen Lol p 5 was isolated from a cDNA expression library. The new isoform, Lol p 5C, shares 95% amino acid sequence identity with Lol p 5A. Both isoforms demonstrated shared antigenic activity but different allergenic activities. Recombinant Lol p 5C demonstrated 100% IgE reactivity in 22 rye grass pollen sensitive patients. In comparison, recombinant Lol p 5A showed IgE reactivity in less than 64% of the patients. Therefore, Lol p 5C represents a novel and highly IgE-reactive isoform allergen of rye grass pollen.

Key words: Lol p 5C; Pollen allergen; cDNA cloning; Isoform; Human IgE; *Lolium perenne*

1. Introduction

Grass pollen sensitisation is a well known cause of hayfever and allergic asthma [1], affecting 25–30% of the population in developed countries [2]. In Australia, rye grass (*Lolium perenne*) pollen is the major contributor to airflora as it is extensively sown as an agriculturally viable livestock feed crop and is capable of producing up to half a tonne of pollen per hectare [3]. Major or dominant allergens of rye grass pollen include Lol p 1 and Lol p 5, and 90% of individuals allergic to rye grass pollen demonstrate IgE reactivity to both of these allergens [4]. Immunocytological studies have shown that Lol p 1 is located in the cytosol of rye grass pollen while Lol p 5 is localised in starch granules [1,5]. Lol p 5's unique localisation in starch granules, and release of such granules into the atmosphere as allergenic micronic particles, have prompted us to identify its role as a trigger of allergic asthma [6,7]. These findings were also confirmed for the timothy grass allergens, Phl p 1 and Phl p 5 [8]. However, the biological function of grass pollen allergens largely remains unknown with the exception of Cyn d 7, from Bermuda grass pollen, which we have recently identified and immunologically characterised as a calcium-binding allergen [9].

Different isoforms of each allergen are identified by a letter designation following the group number (i.e. Lol p 5A, Lol p 5B etc.), according to the International Union of Immunological Societies (IUIS) [10]. Indeed, it has been shown that when crude rye grass pollen extract is separated by two-dimensional (2D)-PAGE and replica immunoblots screened with Lol p 1- and Lol p 5-specific monoclonal antibodies, Lol p 1 contains four isoforms while Lol p 5 contains eight [11]. Isoforms of a protein are essentially the same proteins, often with the same molecular mass (M_r) but with different isoelectric points (p*I*), that exhibit slight physicochemical and immunological differences and share a significant sequence identity at both the nucleic and amino acid level. Cloning of cDNAs encoding Lol p 1 [12,13], and Lol p 5 [14,15], as well as those from other grass pollen [16–18], has shown that each allergen consists of a family of similar but distinguishable sequences. These sequence differences highlight the need to assess the relative potency or clinical significance of all isoforms and identify common and/or unique IgE binding epitopes [19]. Such characterisation studies are mandatory for the design of standardised and effective diagnostics and production of hypoallergenic variants for immunotherapy.

In this paper, we report the cDNA cloning and immunological characterisation of a novel isoform of rye grass pollen allergen Lol p 5, designated Lol p 5C according to IUIS [10], demonstrating high frequency and intensity of IgE reactivity. It is anticipated that such cloning studies will permit the production of hypoallergenic mutants of Lol p 5C, in which B cell epitopes are abrogated, allowing its use as a safer and more effective immunotherapy reagent, without the current risk of IgE-mediated systemic reactions [20].

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[1] The nucleotide and amino acid sequences of Lol p 5C have been submitted to the EMBL/GenBank databases under the accession number AJ243504.

Abbreviations: aa, amino acid(s); ANGIS, Australian National Genomic Information Service; CRPE, crude rye grass pollen extract; IEF, isoelectric focussing; IPG, immobilised pH gradient; IUIS, International Union of Immunological Societies; mAb, monoclonal murine antibody; M_r, molecular mass; NC, nitrocellulose; nLol p 5, natural Lol p 5; nt, nucleotide(s); ORF, open reading frame; p*I*, isoelectric point; RAST, radioallergosorbent test; rLol p 5, recombinant Lol p 5; TBP, tributyl phosphine; 2D, two-dimensional

0014-5793/99/$20.00
PII: S0014-5793(99)01576-8

2. Materials and methods

2.1. Human sera and murine monoclonal antibodies

Human sera were obtained, with oral and written consent, from grass pollen allergic and non-allergic individuals attending the Allergy Clinic at the Alfred Hospital. Allergic status was defined by a history of grass pollen allergy, positive skin prick test to rye grass pollen and the presence of serum IgE specific to rye grass pollen, as determined by radioallergosorbent test (RAST). Lol p 5-specific monoclonal antibody FMC A7 (12.33) was produced at the Flinders Medical Centre (Adelaide, S.A., Australia) in 1983 [21] by immunisation of mice with crude rye grass pollen extract and screening for monoclonal murine antibodies (mAbs) which recognised human IgE binding proteins.

2.2. Construction and screening of cDNA library

Total RNA was extracted from rye grass pollen (*L. perenne*) using the method of Chomczynski and Sacchi [22]. Polyadenylated RNA was purified using an oligo(dT) affinity column (Pharmacia, Sweden) and a cDNA library constructed in the expression vector λ ZAP II (Stratagene, CA, USA) using a Time-Saver cDNA synthesis kit (Pharmacia, Sweden) following manufacturer's instructions. Screening of the cDNA library was accomplished by taking plaque lifts and, following denaturation and neutralisation, probing with ^{32}P random primed (Bresatec, Australia) cDNA clone encoding Lol p 5A, following established methods [23]. After washing and exposure of filters to X-ray film, positive clones were identified, excised from the master plate and different dilutions of clones were re-plated and re-probed to isolate individual and therefore, pure plaques.

2.3. Isolation and sequencing of cDNA clones

cDNA inserts from λ ZAP II clones were sub-cloned into pBluescript SK vectors (Stratagene, CA, USA) by co-infecting *Escherichia coli* SOLR cells with the λ ZAP II phage and f1 helper phage, according to the manufacturer's instructions. DNA sequence was determined by the dideoxy chain termination method [24] using a T7 DNA sequencing kit (Pharmacia-LKB, Uppsala, Sweden) according to the manufacturer's instructions and also confirmed by automated sequencing (Applied Biosystems, USA). Sequence analysis, including amino acid (aa) deduction, was performed using the software program DNA Strider (Commissariat a l'Energie Atomique, France). The nucleotide (nt) and aa sequences of Lol p 5C were searched for comparison with other sequences in the Australian National Genomic Information Service (ANGIS) database which incorporates databases from the following major sources: GenBank, EMBL and NBRF nucleic acid libraries, NBRF PIR protein and Swiss-Prot libraries. The nt and deduced aa sequences of the Lol p 5C cDNA have been submitted to the EMBL/GenBank databases under the accession number AJ243504.

2.4. Expression and purification of rLol p 5C

The cDNA inserts encoding Lol p 5C and Lol p 5A were ligated, in frame, into the pProEx HT expression vector (Life Technologies, USA) following the manufacturer's instructions and transformed into competent cells of *E. coli* strain DH5α (Life Technologies, USA). In order to determine the optimal conditions for expression of recombinant proteins (i.e. rLol p 5C and rLol p 5A), small scale time-course induction of pProEx-Lol p 5A/C was performed as per the manufacturer's instructions. The 4 h post-induction time point was chosen as the optimal induction time. Total bacterial proteins were obtained B-PER bacterial protein extraction reagent (Pierce, USA) following the manufacturer's instructions. rLol p 5A/C allergens were purified from the crude bacterial lysates by mAb A7-affinity chromatography (ImmunoPure protein G IgG Orientation kit; Pierce, USA), following the manufacturer's instructions.

2.5. SDS and 2D gel electrophoresis

Pollen of rye grass (*L. perenne*) was purchased from Greer Laboratories (Lenoir, NC, USA) and total proteins extracted as described previously [25]. Total pollen and bacterial proteins (30 μg/lane), purified rLol p 5A/C proteins (5 μg/lane) and pre-stained molecular weight markers (Novex, CA, USA; 5 μl/lane) were resolved on 12.5% 'mini' SDS-PAGE gels in a Xcell II Mini-Cell (Novex, CA, USA), following the manufacturer's instructions. The first dimension, or isoelectric focussing (IEF), of crude rye grass pollen extract (CRPE) was performed with immobilised pH gradient (IPG) strips and electrophoresed on Multiphor (Pharmacia, Sweden), following the manufacturer's instructions. The second dimension (i.e. SDS-PAGE) was performed using 4–15% gradient 'mini-gels' as described above. Gels were either stained for total proteins with Coomassie brilliant blue [25] or transferred onto nitrocellulose (NC) membrane for immunological studies as described below.

2.6. Immunological analysis and inhibition studies

SDS-PAGE gels containing total grass pollen proteins, purified rLol p 5A/C or IEF resolved CRPE were transferred onto NC using Xcell II blotting apparatus (Novex, CA, USA), following the manufacturer's instructions. Immuno-dot-blots involved the pipetting of CRPE (1 μg/μl/dot) and affinity-purified natural Lol p 5 and rLol p 5A/C proteins (0.2 μg/μl/dot) onto NC membrane grids and allowing them to air-dry. Following immobilisation of proteins onto NC membranes, all blots were blocked in 10% w/v skim milk powder in PBS and probed for either mAb FMC A7 (cell culture supernatant diluted 1:9, 5 ml/blot) or human IgE binding (dot-blot: individual sera diluted 1:4, 1 ml/blot; SDS-PAGE blot: serum pool of 10 grass pollen allergic individuals diluted 1:4, 5 ml/blot) following established protocols [25]. Negative control blots were probed with a serum pool of three non-allergic individuals while 'no serum/mAb' controls were probed with the secondary and/or tertiary detection antibodies only. Negative control blots failed to demonstrate any antibody binding.

Inhibition studies involved NC blots of total rye grass pollen proteins, incubated in a 10 grass pollen allergic patient serum pool (diluted 1:4, 5 ml/blot) either in the absence (positive control) or pre-incubated for 1 h at room temperature in the presence of potential inhibitors of IgE binding [19,25]. Inhibitors included affinity-purified rLol p 5A and rLol p 5C (100 μg/5 ml serum/blot). 2D blots used the same serum pool (diluted 1:4, 5 ml/blot) and affinity-purified rLol p 5A/C were used as the inhibitors (50 μg/5 ml serum/blot). Similarly, NC blots containing SDS-PAGE resolved CRPE and affinity purified rLol p 5A/C, were probed with mAb FMC A7 (cell culture supernatant diluted 1:9, 2.5 ml/blot) and affinity-purified rLol p 5A/C were used as the inhibitors (50 μg/2.5 ml/blot).

3. Results and discussion

3.1. Molecular cloning and characterisation of Lol p 5C

Seven positive clones were identified from the rye grass pollen cDNA expression library. All positive clones were recognised by IgE antibodies from a pool of sera taken from grass pollen allergic patients while no signal was detected when plaques were screened with non-allergic human sera and no serum antibody controls. The ability of these clones to hybridise to Lol p 5A and bind to specific IgE antibodies in the sera of grass pollen sensitive individuals identified these as encoding Lol p 5 allergens. Consequently, in order to determine whether any of these clones encoded novel allergen isoforms of Lol p 5, they were sub-cloned into the pBluescript SK vector to permit DNA sequencing. Although sequentially different to Lol p 5A, a number of the positive clones did not encode full-length proteins. Also, some of the clones were identical to Lol p 5A and were therefore omitted from further study. However, one full-length clone, clone 4, demonstrated some sequence differences from Lol p 5A throughout the molecule and in particular in the 3′ untranslated region (Fig. 1). The nt sequence of cDNA clone 4 has an open reading frame (ORF) of 906 base pairs (bp) starting with an ATG initiation codon at position 32 and terminating with a TGA stop codon at position 935 (Fig. 1). It also contains a complete 3′ untranslated region containing a poly $(A)^+$ tail. The ORF encodes a mature protein of 276 aa (~27.5 kDa) with a calculated p*I* of 6.5. In comparison, Lol p 5A cDNA encodes a mature protein of 276 aa (~27.3 kDa) with a calculated p*I* of 6.7 while Lol p 5B encodes a 314 aa protein (~31.3 kDa) with a p*I* of 7.1 [14,15]. Comparison of the deduced aa se-

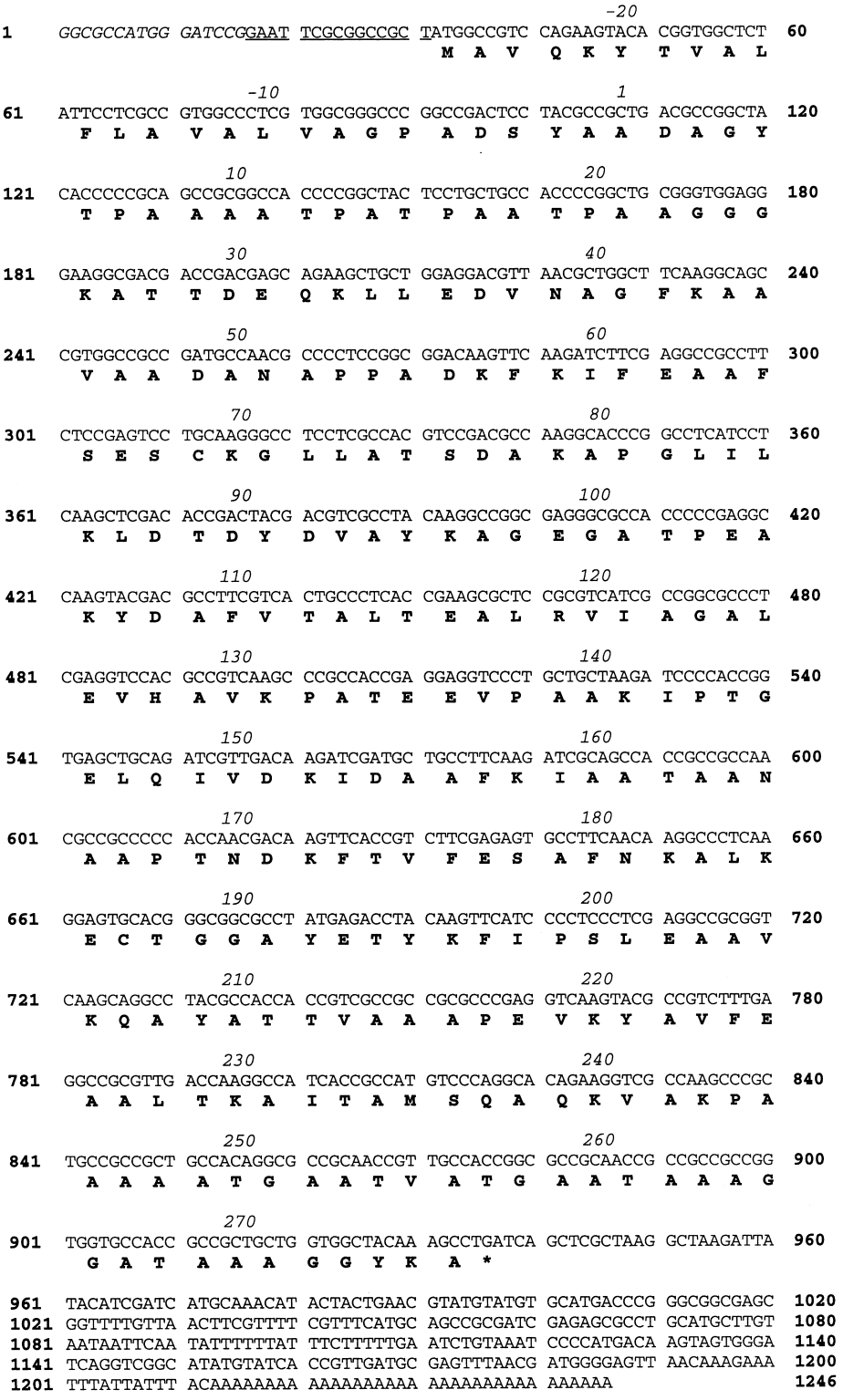

Fig. 1. Nucleic and deduced aa sequences of Lol p 5C. Numbers on margins correspond to bp positions. Numbers above the nt sequence refer to aa positions. Negative numbers refer to the signal peptide, which is cleaved off from the mature protein. The 5′ underlined sequence represents the adapter used during cDNA synthesis and the italic sequence refers to the multiple cloning site of the vector DNA sequence. Asterisk corresponds to the TGA stop codon.

quence of clone 4 with that of Lol p 5A and Lol p 5B indicates a high level of similarity (Fig. 2). Clone 4 shares 95% sequence identity with Lol p 5A and 56% identity with Lol p 5B, while there is only 56.6% identity between Lol p 5A and Lol p 5B (Table 1). Therefore, clone 4 encodes a novel Lol p 5 isoform that is more similar to Lol p 5A than Lol p 5B. However, although clone 4 is highly similar to Lol p 5A, clone 4 also possesses 11 unique aa residues among which is an

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5C    MAVQKYTVALFLAVALVAGPADSYAADAGYTPAAATPATPAATPAAG--   48
5A    .....Y.............A........T..AA....T..ATPAAA--   48
5B    .....H.............A........A..TP....A..TAATPATP   50

5C    ----------GGKATTDEQKLLEDVNAGFKAAVAADANAPPADKFKIFEA   88
5A    ----------G.....D....L.DV..........A.NA.....F.I.EA   88
5B    ATPATPAAVPS.....E....I.KI..........A.VV.....Y.T.VE  100

5C    AFSESCKGLLATS------DAKAPGLILKLDTDYDVAYKAGEGATPEAKY  132
5A    A.SESSKGLLATS------AAKAPG.IP...TAYDV..K.AE........  132
5B    T.GTATNKAFVEGLASGYADQSKNQ.TS...AALKL..E.AQ........  150

5C    DAFVTALTEALRVIAGALEVHAVKPATEEVPAAKIPTGELQIVDKIDAAF  182
5A    ..F.TA..........A.........T...PAAK..TG.L.IV..I...F  182
5B    ..Y.AT..........T.........A...KVGA..AA.V.LI..V...Y  200

5C    KIAATAANAAPTNDKFTVFESAFNKALKECTGGAYETYKFIPSLEAAVKQ  232
5A    KI.........T........SA..K.LNECT.G..ET.....S.E.....  232
5B    RT.........A........NT..N.IKVSL.A..DS.....T.V.....  250

5C    AYATTVAAAPEVKYAVFEAALTKAITAMSQAQKVAKPAAAAATGAATVAT  282
5A    ...ATV.T......A.F.A..T..I...TQ.Q.AGK.....ATGAATV.T  282
5B    ...AKQ.T......T.S.T..K..V...SE.E.EAT.....TATPTPA.A  300

5C    GAAT--------------------AAAGGATAAAGGYKA              301
5A    G.AT--------------------...GAAT.......A              301
5B    T.TATPAAAYATATPAAATATATP...TATP.......V              339
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Fig. 2. Comparison of the deduced aa sequence of Lol p 5C with other cloned and sequenced Lol p 5 isoforms. Numbers refer to aa residues. Gaps represented by dashes (–) have been introduced to ensure maximum homology. Lol p 5A and Lol p 5B deduced aa sequences are from reference [14] and have the accession numbers M59163 and L13083, respectively. Amino acid residues common to all isoforms are represented by dots (·). Underlined aa residues of Lol p 5C sequence represent residues common to only Lol p 5A and Lol p 5C. Italic aa residues of Lol p 5C sequence represent residues common to only Lol p 5B and Lol p 5C. Bold aa residues of Lol p 5C sequence indicate residues unique to Lol p 5C.

additional cysteine residue (Fig. 2). Thus, it is assumed that clone 4 would encode a protein that may adopt a totally different secondary and/or tertiary structure (i.e. via a disulfide bond) to that of Lol p 5A (and also Lol p 5B) and may also have different allergenic activity. Consequently, clone 4 was named Lol p 5C, in accordance with IUIS nomenclature [10], and its sequence submitted to the EMBL/GenBank Nucleotide Sequence Databases under accession number AJ243504.

3.2. Immunological characterisation of Lol p 5C

The cDNA encoding Lol p 5C was sub-cloned into the pProEx HT bacterial expression vector to express rLol p 5C (rLol p 5C) and to assess its immunological properties. Fig. 3A shows the time-course of induced and non-induced *E. coli* cells containing the Lol p 5C cDNA-vector. There is no expression of Lol p 5C in non-induced cells (Fig. 3A, lanes 1, 3, 5, 7). However, 2 h after induction, accumulation of rLol p 5C is evident at ~27 kDa (Fig. 3A, lane 4). Expression peaks at 4 h (Fig. 3A, lane 6) and declines significantly by 8 h post-induction (Fig. 3A, lane 8). Therefore, the optimal expression for rLol p 5C was chosen to be 4 h post-induction.

In order to assess if rLol p 5C was immunologically reactive, replica gels of the time-course were blotted onto NC and probed with patient serum IgE or the Lol p 5-specific monoclonal antibody, FMC A7 (Fig. 3B,C, respectively). Indeed, rLol p 5C was IgE reactive and corresponded to the protein band observed on the Coomassie-stained gel as rLol p 5C (Fig. 3B). No IgE binding was detected with serum taken

Table 1
Percentage identity between the deduced aa sequences of Lol p 5C and other Lol p 5 isoforms, as shown in Fig. 2

	Lol p 5A	Lol p 5B	Lol p 5C
Lol p 5A		56.6%	95%
Lol p 5B	56.6%		56%

from non-allergic individuals and no serum controls. Once again, optimal IgE binding was observed for the 4 h time-point (Fig. 3B, lane 8). Although Lol p 5A demonstrates specific binding to mAb FMC A7, Lol p 5B does not [14]. Therefore, it was of great interest to determine whether the epitope responsible for mAb binding was conserved in Lol p 5C. Fig. 3C demonstrates intense mAb FMC A7 binding to rLol p 5C; especially at the 4 h time-point (Fig. 3C, lane 6). No secondary antibody binding was detected when mAb FMC A7 was omitted from the immunoblotting procedure.

We have recently reported the IgE and mAb FMC A7 binding epitopes of Lol p 5A [19]. The monoclonal antibody recognised Lol p 5A (aa 49–60), an epitope which has a 92% identity with Lol p 5C (aa 49–60) and only 67% identity with Lol p 5B (i.e. Lol p 5C (aa 74–85) in Fig. 2) [19]. On the other hand, a number of IgE binding epitopes were detected in Lol p 5A, with two epitopes demonstrating high affinity to human IgE. One of these was, once again, Lol p 5A (aa 49–60) and the other was Lol p 5A (aa 265–276) (i.e. Lol p 5C (aa 290–301) in Fig. 2) [19]. The latter epitope shows 92% identity with Lol p 5C, while only 67% identity exists between Lol p 5A and Lol p 5B (Fig. 2). However, the aa residues critical for IgE binding to this epitope have been confined to Lol p 5A (aa 272–275), an area demonstrating 100% identity with Lol p 5B and Lol p 5C (i.e. Lol p 5C (aa 297–300) in Fig. 2).

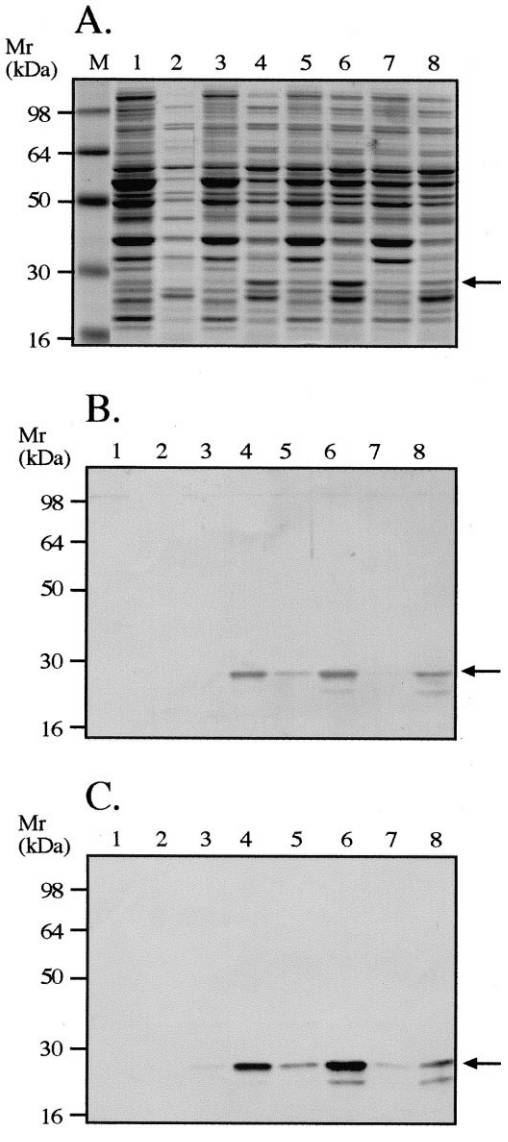

Fig. 3. Bacterial expression and immunoblot analysis of rLol p 5C. Time-course of bacterial protein expression of cells containing cDNA encoding Lol p 5C and analysis on a gel stained with Coomassie (A), or transferred onto NC and probed with a serum pool from grass pollen allergic individuals (B), or Lol p 5-specific mAb FMC A7 (C). M_r markers are in lane M. Samples were taken at 0 (lanes 1, 2), 2 h (lanes 3, 4), 4 h (lanes 5, 6) and 8 h intervals after induction. Odd numbered lanes represent the uninduced culture and even numbered lanes represent the induced culture. Arrows point to the accumulation of rLol p 5C in the induced culture.

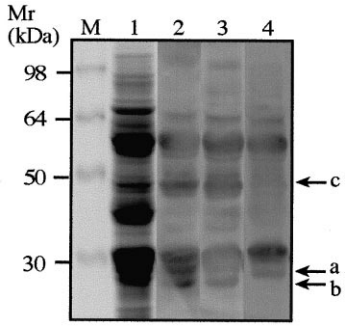

Fig. 4. Inhibition of IgE binding to CRPE by rLol p 5A and rLol p 5C. Total proteins of CRPE were resolved by SDS-PAGE and either stained with Coomassie (lane 1) or transferred onto NC for immunoblot analysis (lanes 2–4). M_r markers are in lane M. NC blots were probed for IgE binding with a serum pool of grass pollen allergic patients only (lane 2), or the same serum pool pre-incubated either with affinity-purified rLol p 5A (lane 3) or rLol p 5C (lane 4). Arrow (a) points to nLol p 5A (i.e. IgE binding of this protein inhibited by rLol p 5A) and arrow (b) points to nLol p 5C (i.e. IgE binding of this protein inhibited by rLol p 5C). Arrow (c) points to a potential dimer of nLol p 5C or related proteins (i.e. IgE binding of these proteins inhibited by rLol p 5C).

3.3. *Immunological similarity between Lol p 5A and Lol p 5C proteins*

Fig. 4 demonstrates CRPE resolved by SDS-PAGE (lane 1) and NC blots probed for total IgE binding (lane 2) with serum pool from grass pollen allergic patients, and the serum pool pre-incubated with either rLol p 5A or rLol p 5C (lanes 3 and 4, respectively). Total IgE recognised all major allergens of CRPE, including the usual 2 or 3 protein bands corresponding to natural Lol p 5 (nLol p 5) around the 28–30 kDa region [19]. However, pre-incubation of the serum pool with purified rLol p 5A, reduced IgE binding to a higher band of nLol p 5 (Fig. 4, arrow a). On the other hand, pre-incubation of the serum pool with rLol p 5C reduced IgE binding to the lower nLol p 5 protein band (Fig. 4, arrow b) and, more interestingly, to a group of 2 or 3 allergens around the 50 kDa region (Fig. 4, arrow c) which may represent dimer forms of Lol p 5C (or related proteins). Our proposed explanation for this observation in regard to the 50 kDa allergens comes from our results with the same experiment conducted with CRPE resolved by 2D-SDS-PAGE (Fig. 5). In the 2D gels, occurence of the 50 kDa allergens was less obvious, perhaps due to presence of urea in the sample buffer. Indeed, addition of urea to the CRPE samples resolved by SDS-PAGE decreased both the protein amount and IgE reactivity of proteins in this

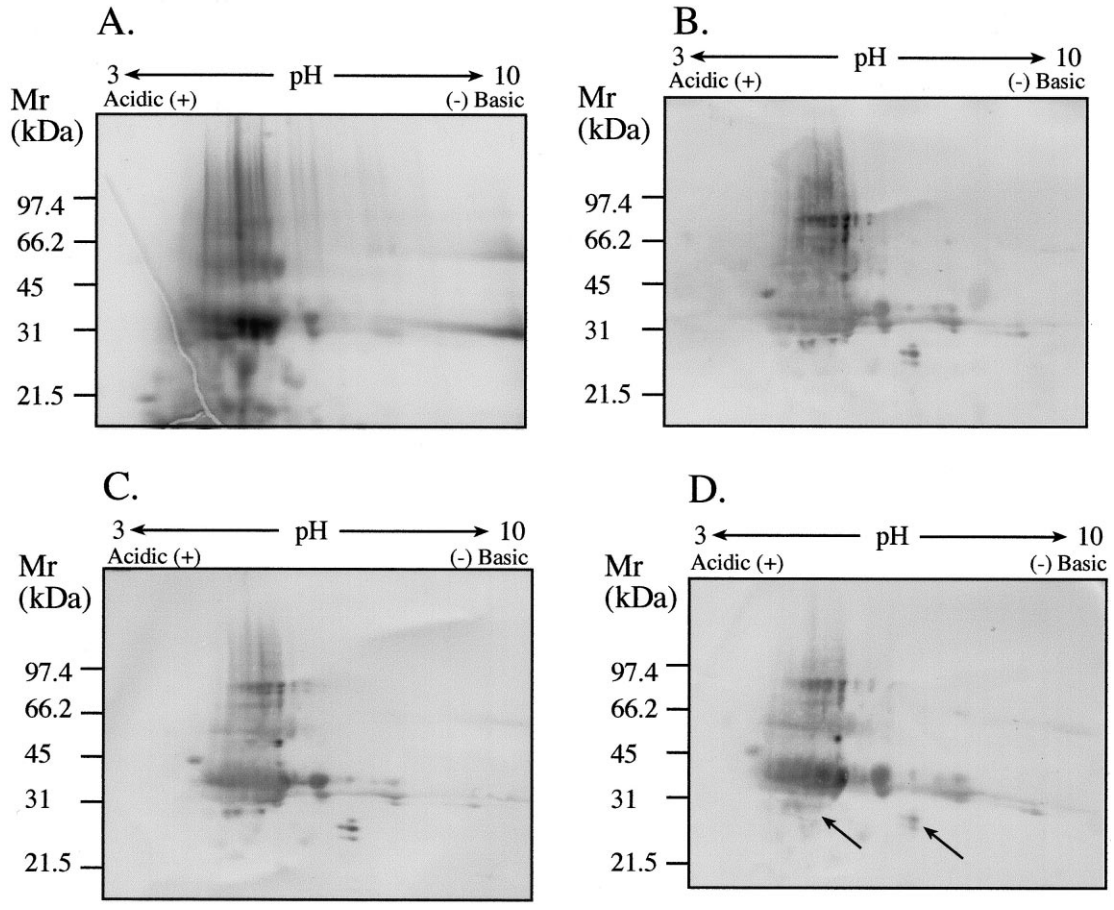

Fig. 5. Inhibition of IgE binding to CRPE resolved by 2D-PAGE. Gels were either stained with Coomassie (A) or transferred onto NC for immunoblot analysis (B–D). NC blots were probed for IgE binding with a serum pool of grass pollen allergic patients only (B), or the same serum pool pre-incubated either with affinity-purified rLol p 5A (C) or rLol p 5C (D). Arrows point to nLol p 5C, or related, proteins to which IgE binding was inhibited by rLol p 5C.

region (data not shown), suggesting that Lol p 5C may occur as a dimer in the natural extract.

In relation to 2D-PAGE resolved nLol p 5 at the 30 kDa region, inhibition of IgE binding was very slight with rLol p 5A (Fig. 5C), due to low level of IgE reactivity of rLol p 5A. However, rLol p 5C reduced IgE binding to several protein spots occupying the relevant regions in terms of both M_r and p*I* (Fig. 5D, arrows). No IgE binding was detected with sera taken from non-allergic individuals. Therefore, it appears that rLol p 5A and rLol p 5C may have different IgE binding epitopes. In another experiment, looking at the antigenic similarity between rLol p 5A and rLol p 5C, both allergen isoforms inhibited mAb FMC A7 binding to Lol p 5A and Lol p 5C as well as to nLol p 5 (Fig. 6), suggesting that both proteins possess the same mAb FMC A7 binding epitope(s).

3.4. Biological and clinical significance of Lol p 5C

Unfortunately, cloning and sequencing of Lol p 5C did not provide any new information regarding the biological function or role of this allergen group in rye grass pollen. Unlike allergens from other sources (e.g. house dust mites etc.), determination of biological function of pollen allergens with database sequence search/comparisons has been largely unsuccessful. However, we have recently reported, for the first time for a grass pollen allergen, the cloning and immunological characterisation of a novel calcium-binding Bermuda grass pollen allergen, designated Cyn d 7 [9]. It is anticipated that the specific expression of grass allergens in the pollen grain indicates their possible involvement with pollen germination and pollen tube growth.

In order to obtain a more complete picture of rLol p 5C's IgE reactivity, and therefore its clinical significance in relation to rLol p 5A, we obtained sera from 22 patients allergic to rye

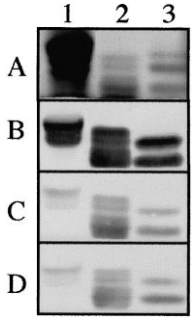

Fig. 6. Inhibition of mAb FMC A7 binding to CRPE and affinity-purified rLol p 5A and rLol p 5C. SDS-PAGE resolved CRPE (lane 1) and affinity-purified rLol p 5A (lane 2) and rLol p 5C (lane 3) either stained with Coomassie (A) or transferred onto NC for immunoblot analysis (B-D). NC blots were probed for mAb binding only (B), or pre-incubated either with affinity-purified rLol p 5A (C) or rLol p 5C (D) then probed for mAb binding.

Table 2
Frequency and strength of IgE binding, from sera of 22 allergic and three non-allergic individuals, to dot blots of CRPE (1 mg/dot) and affinity-purified natural (n; 0.2 mg/dot) and recombinant (r; 0. mg/dot) Lol p 5 isoforms

Allergen	Frequency of IgE binding (%)				
	Sera from allergic individuals ($n=22$)				Sera from non-allergic individuals ($n=3$)
	Strong	Moderate	Weak	None	
CRPE	68.2	27.3	4.5	0	0
nLol p 5	72.7	22.7	4.6	0	0
rLol p 5A	9.1	31.8	22.7	36.4	0
rLol p 5C	72.7	22.7	4.6	0	0

Strength of IgE binding was assessed visually when compared to the control background.

grass pollen, possessing specific IgE to nLol p 5, to probe dot-blots of affinity-purified rLol p 5A, rLol p 5C, nLol p 5 and CRPE (Table 2). All of the patient sera showed IgE reactivity to CRPE, and 68.2% were categorised as demonstrating strong IgE binding. 72.7% of the patient sera showed strong IgE binding to nLol p 5 and rLol p 5C. In contrast, only 9.1% of the patient sera showed strong IgE binding to rLol p 5A and 36.4% gave no IgE binding to rLol p 5A (Table 2). Therefore, Lol p 5C appears to be a more clinically significant allergen isoform of Lol p 5.

Recently, site-directed mutagenesis has been successful in the reduction of IgE-reactivity of a major house dust mite allergen Der p 2 [26] and a major birch pollen allergen, Bet v 1 [27]. With the latter, analysis of the deduced aa sequences of the different isoforms of Bet v 1 allergen, each with a different IgE-reactivity, identified six critical aa residues [27]. Single aa substitutions at the six key aa residues of Bet v 1 resulted in a Bet v 1 mutant with extremely low reactivity to serum IgE from birch pollen allergic patients [27]. However, proliferation assays of allergen specific T-cell clones demonstrated that T-cell recognition was maintained. Therefore, such studies highlight the importance of cloning and immunological characterisation of allergen isoforms in understanding the molecular basis of IgE reactivity of allergens and, therefore, provide information in the design of novel hypoallergenic mutants for the treatment of grass pollen induced allergies [20].

Acknowledgements: The authors wish to thank Dr B. Walsh and his group from the Australian Proteome Analysis Facility for their help with 2D analysis; Mr M. Burton for the provision of mAb FMC A7 cell supernatant and the Australian National Health and Medical Research Council and the Australian Allergy Foundation for their financial support.

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