

31381

## Two-Dimensional Gel Electrophoretic Analyses of Kentucky Bluegrass and Rye Grass Pollen Allergens

Detection with a Murine Monoclonal Anti-*Poa p I* Antibody and Amino Terminal Amino Acid Sequence of *Poa p I* Allergen<sup>1</sup>

Abul K.M. Ekramoddoullah

Pacific Forestry Centre, Forestry Canada, Victoria, Canada

**Abstract.** Allergenic extracts of Kentucky bluegrass (*Poa pratensis*) and rye grass (*Lolium perenne*) pollen were shown by 2-dimensional (2-D) gel electrophoresis to consist of several hundred protein components. The pollen extracts of the two related grasses had unique 2-D gel patterns. Two major grass pollen allergens, *Poa p I* and *Lol p I*, and their isoforms (i.e. isoallergens) were detected and localized on 2-D gels by immunoblotting with anti-*Poa p I* antibody mAb 60. *Poa p I* isoallergens were less acidic than *Lol p I* isoallergens. The relative proportion of four *Poa p I* isoallergens was (in decreasing pI): A, 13%; B, 37%; C, 35%; and D, 15%. The amino terminal amino acid sequences of the two major isoallergens of *Poa p I* were identical. However, the amino acid composition of these isoallergens showed enough differences to account for their charge differences. The amino terminal amino acid sequence of two major *Poa p I* isoallergens had a 70% homology in 20 amino acid overlap with the previously published amino terminal amino acid sequence of rye grass pollen allergen R-7.

### Introduction

Grass pollens, particularly Kentucky bluegrass (KBG), are major hay fever-causing agents in the prairies and western regions of North America. The common form of treatment for this disease consists of a series of injections of a crude extract consisting of a heterogeneous mixture of constituents of the offend-

ing pollen. The sensitivity of allergic individuals to these various components varies widely and consequently the treatment itself may sensitize individuals to components to which they were not originally sensitive. It might be desirable to treat each individual with purified allergen(s) to which they are actually sensitive. In the past, a combination of several physicochemical methods have been used to purify allergens. These methods are painstakingly laborious and time consuming and it is seldom commercially viable to isolate pure allergens in large quantities. However, the feasibility of isolating large amounts of pure allergens is demonstrated by the recent application of hybridoma [1] and cDNA [2, 3] technologies in the isolation and characterization of pollen allergens.

<sup>1</sup> The salary support of the technician was provided by the British Columbia Ministry of Forests under a Memorandum of Understanding with Forestry Canada.

The sequence data has been submitted to GenBank (Accession No. A33086; Protein Identification Resource database National Biomedical Research Foundation, Washington, D.C.), Los Alamos National Laboratory, Los Alamos, NM 87545, USA.

Thus, it is envisaged that purified allergens can be commercially prepared by recombinant DNA technology and that these could be used for hyposensitization purposes. Successful cloning of genes coding for allergens is greatly simplified if some amino acid sequence information of the allergen is available. This type of information for a given allergen may be obtained by two-dimensional (2-D) gel electrophoresis without having to purify the allergen from the crude extract.

In a recent study [4], *Poa p* I allergen was isolated from KBG pollen extract by a reverse immunosorbent prepared with a murine monoclonal antibody (mAb) 290-A-167 specific for a rye grass pollen allergen *Lol p* I. The allergenicity of *Poa p* I was confirmed by its reactivity towards IgE antibodies present in sera of KBG-sensitive patients [4, 5]. *Poa p* I was found to consist of a predominantly acidic 35.8-kD component and a basic 33-kD component. Monoclonal antibodies produced against *Poa p* I resulted in antibodies with one group reacting primarily to the 35.8-kD and to a lesser extent the 33-kD components, and the other group reacting to the 35.8 kD component only. The mAbs also recognized rye grass pollen allergen *Lol p* I. This study reports the utilization of anti-*Poa p* I mAb 60 which binds predominantly the 35.8-kD component and 2-D gel electrophoresis to obtain N-terminal amino acid sequence information of *Poa p* I and the reactivity of rye grass pollen allergens towards mAb 60.

## Materials and Methods

### Preparation of KBG-R and Rye-R

The non-dialyzable components of KBG (*Poa pratensis*) and rye grass (*Lolium perenne*) pollen (Hollister Stier Laboratory, Mississauga, Ont., Canada) were prepared by extracting ether-defatted pollen with distilled water for 4 h at room temperature, followed by dialysis through Spectropor membrane tubing (molecular weight cutoff, 6,000–8,000; Spectrum Medical Industries, Inc., Los Angeles, Calif., USA). The retentate fractions were designated as KBG-R and Rye-R, respectively.

### Production and Purification of mAb 60

mAb 60 was one of the murine mAbs produced against KBG allergen *Poa p* I [5]. mAb 60 was of the IgG1 isotype and possessed  $\kappa$  light chains and binds strongly to the 35.8-kD and to a lesser extent 33-kD components of *Poa p* I [4]. The production and purification of mAb 60 has recently been described [5]. Briefly, female BALB/c mice were immunized with *Poa p* I in Freund's complete adjuvant. The immune spleen cells were fused with NS1 myeloma cells. Clones of hybridoma cells secreting mAb 60 were expanded in vitro and then propagated in the peritoneum of BALB/c female

mice. The mAb was purified from the ascitic fluid by gel filtration on AcA-44 Ultrogel.

### 2-D Gel Electrophoresis of KBG-R and Rye-R

The 2-D gel electrophoresis was carried out according to the recent method described by Hochstrasser et al. [6] with minor modifications. Water used in this and subsequent procedures was purified by passing double distilled water over a Milli-Q water purification system (Millipore Canada Ltd, Toronto, Ont., Canada). First, several stock solutions were prepared: solution A contained 30 g of acrylamide (Bio-Rad Laboratories, Richmond, Calif., USA), 0.8 g diacrylpiperazine (Bio-Rad), and water to make up to 100 ml; solution B consisted of 0.3 g cholamidopropyltrimethylhydroxypropanesulfonate (CHAPS; Sigma Chemical Corporation, St. Louis, Mo., USA), 0.1 ml of NP-40 (Sigma), and 0.9 ml of water; solution C consisted of 182 g of Tris base (Bio-Rad), adjusted to pH 8.8 with HCl, and water to make 1 liter; solution D contained 1 g of ammonium persulfate (Bio-Rad) and 10 ml of water; solution E contained 40 ml of 10% SDS (Bio-Rad), 20 ml of Tris-HCl (0.5 M, pH 6.8), 8 ml of 0.05% bromophenol blue, and 72 ml of water; solution F contained 0.1 g of dithiothreitol (Bio-Rad), 0.4 g of CHAPS, 54 g of urea (Serva, Heidelberg/New York), 0.5 ml ampholine 3.5–10 (Bio-Rad), and 6.5 ml of water. The isoelectric focusing (IEF) gels were prepared by mixing 10 g of urea, 7 ml of water, 2.5 ml of solution A, 0.8 ml of ampholytes 3–10, 0.2 ml of ampholytes 5–7, 1 ml of solution B, 0.02 ml of TEMED (Bio-Rad) and 0.04 ml of solution D. After appropriate degassing, IEF gels were polymerized onto tubes (130 × 2 mm); gel heights were usually 120 mm. The IEF was carried out in a Hoefer GT 2 electrophoresis cell (Hoefer Scientific Instruments, San Francisco, Calif., USA). A 0.02-ml volume of sample (containing 200  $\mu$ g of lyophilized KBG-R or Rye-R dissolved in solution F) was loaded onto the gel and carefully overlaid with the catholyte solution, i.e., 20 mM NaOH. The upper tank buffer was filled with the catholyte while the lower tank buffer was filled with the anolyte, 6 mM  $H_3PO_4$ . The IEF was run at room temperature at a precisely controlled voltage: first for 2 h at 200 V, then for 5 h at 500 V, and finally for 16 h at 800 V. The power supply used was Bio-Rad model 3000. IEF gels were removed, briefly rinsed with solution E (0.15 ml/gel), and laid on top of the second-dimension SDS-PAGE gels. Electrophoresis in the second dimension was carried out in Hoefer's multiple slab gel apparatus (Model SE 700). SDS-PAGE gels (13.3%) were prepared by mixing 715 ml of solution A, 440 ml of 1.5 M Tris-HCl (pH 8.8), 495 ml of water, 0.22 ml of TEMED, and 3.8 ml of solution D. The running lower tank buffers consisted 20 g SDS, 120 g Tris base, 576 g of glycine, 4 g of sodium azide, and 20 liters of water; the same buffer without sodium azide was used as the upper tank buffer. SDS-PAGE was run at a constant current of 40 mA/gel. In some experiments, low molecular weight protein standards (Bio-Rad) were included in SDS-PAGE. At the end of the run, gels were stained with silver.

### Scanning, Spot Detection, and Quantitation of Proteins

Silver-stained gels were scanned by a laser scanner (Molecular Dynamics, model No. 110A, Sunnyvale, Calif., USA) interfaced by PDI (Protein Data bases Inc., Huntington Station, N.Y., USA) for the 2-D gel processing software programs PDQ1 and PDQ2. Scanning, detection, and preparation of standard synthetic images from the original images and quantitation were performed according to the PDI instruction manual.

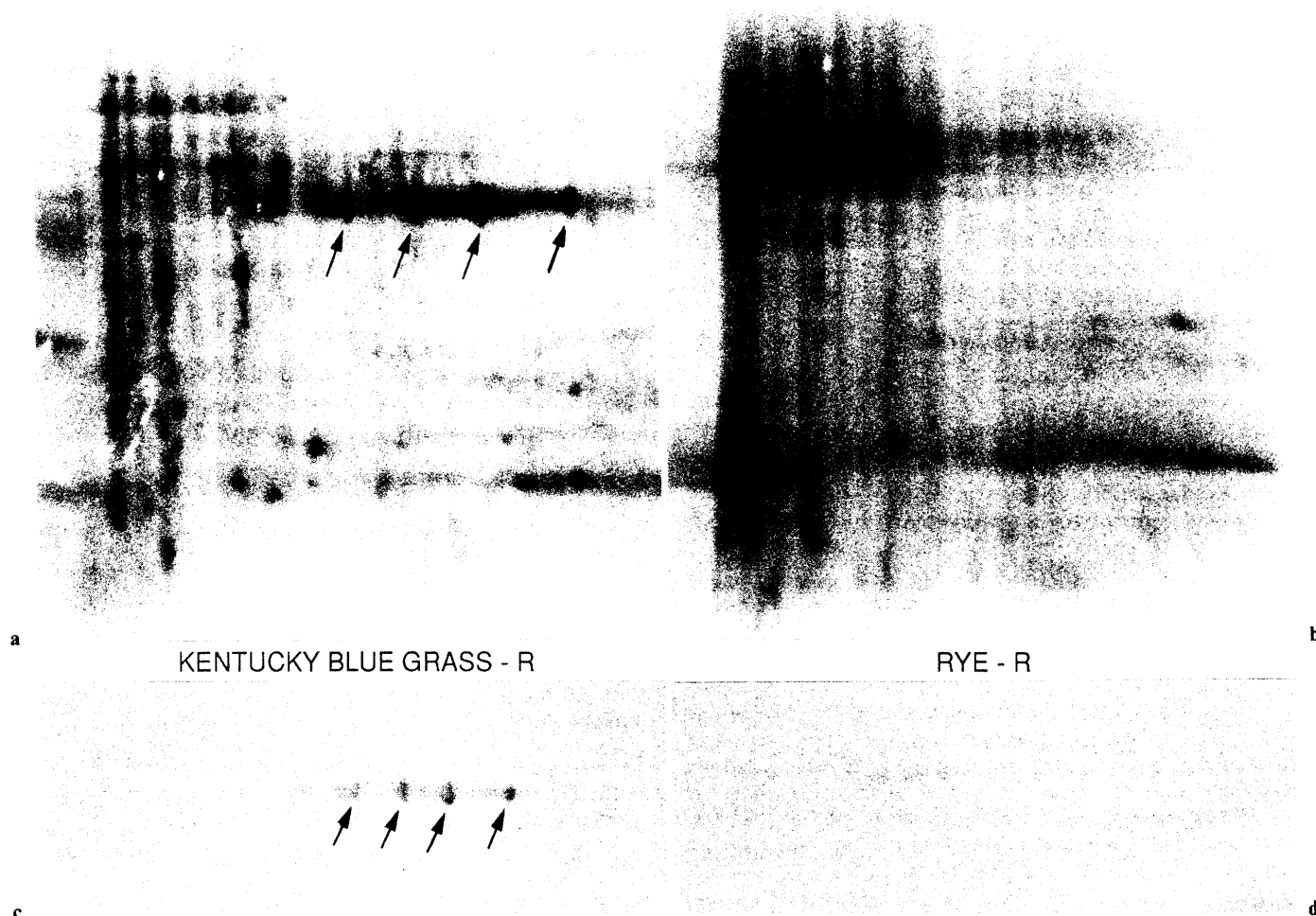


**Fig. 1.** Two-dimensional gel electrophoresis of Kentucky bluegrass-R and rye grass (Rye-R) pollen extracts. **a, b** Gels stained with silver following electrophoresis of the indicated samples. **c, d** The corresponding immunoblot obtained by electrotransfer of the separated proteins from the unstained gels onto nitrocellulose membranes and developed with anti-*Poa p* I antibody mAb 60. Arrows (**a, c**) from left to the right are: spot No. 1 (designated as *Poa p* ID); spot No. 2 (designated as *Poa p* IC); spot No. 3 (designated as *Poa p* IB), and spot No. 4 (designated as *Poa p* IA).

#### Immunoblotting

For immunodetection, 2-D gels were first equilibrated with electrolyte buffer containing 20 mM Tris, 192 mM glycine, and 20% methanol for 5 min. Proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane [7] utilizing the electrolyte buffer and with the electroblotting unit (CBS Scientific Co. model EBU-100, Del Mar, Calif., USA). Electroblotting was run at a constant voltage of 30 V/gel for 18 h at 5°C. Following transfer, the membrane was blocked by incubating the membrane in gelatin [3% in Tris-buffered saline (TBS, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5)] for 30 min at room temperature and then washed with TBS containing 1% Tween 20 (TBST). The membrane was incubated with mAb 60 (5 µg/ml) in the incubation buffer IB (1% gelatin in TBST) for 2 days at room temperature. The membrane was washed with TBS for 5 min (repeated three times) and then incubated with the conjugate of goat anti-mouse IgG (H+L) and alkaline phosphatase (Bio-Rad immunoblot assay kit) diluted 3,000-

fold with IB for 1 h. The membrane was again washed with TBS as above. The alkaline phosphatase color development reagents (Bio-Rad immunoblot assay kit), [5-bromo-4-chloro-3-indolyl]-phosphate-*p*-toluidine salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) were used to develop the color reaction. This was done by mixing 1 ml of NBT solution [30 mg/ml of 70% N,N-dimethylformamide (DMF)] and 1 ml of BCIP solution (15 mg/ml of 70% DMF) into 100 ml of carbonate buffer (0.1 M sodium bicarbonate, 1.0 mM MgCl, pH 9.8). The membrane was incubated in this color development solution for 30 min to produce an insoluble purple product on the membrane. The reaction was stopped by washing the membrane with distilled water for 15 min.

#### Amino Acid Sequence and Composition Analyses

Proteins separated on 2-D gels were electrophoretically transferred to polyvinylidene difluoride (PVDF, Immobilon-P, Millipore) membrane as described by Matsudaira [8] and modified as



terminal amino acid sequence of *Poa p* IA was identical with the sequence of *Poa p* IB or *Poa p* IC for the first 14 amino acid residues, and subsequent amino acid residues could not be ascertained due to lack of material.

Table 1 shows the amino acid composition of the two major isoforms of *Poa p* I allergen. The amino acid composition of the two isoforms were very similar but not identical. The slightly higher percentage of lysine in spot No. 3 is consistent with its relative basicity as shown by its location on the 2-D gels. One of the minor isoforms, *Poa p* ID, had an amino acid composition similar to *Poa p* IC (data not shown). Although a complete amino acid composition for *Poa p* IA could not be obtained with certainty because several amino acids were detected in low amounts, the relative proportion of major amino acids present in this isoform indicated that it had a slightly different amino acid composition from *Poa p* IB and *Poa p* IC; in particular, it had a higher lysine content (data not shown).

## Discussion

Allergenic extracts from two related grass pollens, KBG and rye grass, produced different 2-D gel electrophoretic patterns. These unique patterns and the resolution of 2-D gel electrophoresis along with computation [10] should provide a useful adjunct to existing methods for standardization of allergens [11, 12]. The large number of protein components detected in these grass pollen extracts also illustrates their complex nature and the necessity of establishing the sensitivity of allergic individuals to each of these protein components.

Earlier studies [13–16] indicated a wide range of antigenic cross-reactivity among various grass pollens, including KBG and rye grass pollen. The reactivity of mAb 60 towards the resolved protein components of KBG-R and Rye-R is consistent with this cross-reactivity. However, the results of 2-D gels and the corresponding immunoblot clearly demonstrated that this cross-reactivity was due to two distinct groups of proteins present in these two grass pollens. The results also showed that *Poa p* I and *Lol p* I were different proteins and that they may possess common (or similar) epitopes capable of reacting with mAb 60.

In this study, the minor basic 33-kD (pI 9.1) component of *Poa p* I could not be detected with mAb 60.

**Table 1.** Amino acid composition<sup>1</sup> (residues/molecule<sup>2</sup>) of *Poa p* I isoallergens

| Amino acid | <i>Poa p</i> IC<br>(spot No. 2) | <i>Poa p</i> IB<br>(spot No. 3) |
|------------|---------------------------------|---------------------------------|
| Asx        | 37                              | 35                              |
| Glx        | 34                              | 28                              |
| Ser        | 23                              | 18                              |
| Gly        | 52                              | 48                              |
| His        | 0                               | 4                               |
| Arg        | 13                              | 11                              |
| Thr        | 18                              | 18                              |
| Ala        | 30                              | 30                              |
| Pro        | 17                              | 20                              |
| Tyr        | 13                              | 13                              |
| Val        | 22                              | 22                              |
| Met        | 0                               | 8                               |
| Cys        | 0                               | 0                               |
| Ile        | 14                              | 13                              |
| Leu        | 20                              | 16                              |
| Phe        | 12                              | 12                              |
| Lys        | 30                              | 36                              |

<sup>1</sup> Proteins separated by 2-D gel electrophoresis were electrophoretically transferred to immobilon, stained with Coomassie blue and spots (indicated by the arrow in figure 1) were cut out and subjected to amino acid composition analyses.

<sup>2</sup> The number of amino acid residues was calculated using 36 kD as the molecular weight of *Poa p* I.

This may be due to the loss of the 33-kD component during IEF in the 1st dimension. Thus, a colored basic protein cytochrome c, which was incorporated into the IEF gel to monitor the progress of IEF, was eluted off the gel at the end of each run. Furthermore, a highly basic allergen, *Lol p* IV [16], could not be detected in 2-D gel electrophoresis of Rye-R which was immunoblotted with mAb 12 specific for *Lol p* IV. Modification of IEF in the 1st dimension was not attempted to detect these basic components.

The identical amino terminal amino acid sequence of the two major isoforms of *Poa p* I at first suggested that these proteins are identical and that the charge heterogeneity could have been due to glycosylation or state of amidation. However, amino acid composition analysis clearly showed that these isoforms also differ in their amino acid composition. For example, *Poa p* IB had in total eight more basic amino acids (histidine, arginine and lysine) than *Poa p* IC (table 1); this

is consistent with its relative basicity exhibited on the 2-D gel electrophoretic pattern. Although two hydroxyproline residues were detected in the amino acid sequence analysis, their absence in the amino acid composition analysis was due to their co-elution with glutamic acid during the chromatographic separation of the hydralysates. The amino terminal amino acid sequence of the *Lol p* I identified in this study was not investigated, but the amino terminal sequence obtained for *Poa p* I showed a surprising 70% homology with the N-terminal 20 amino acid sequence of rye grass pollen allergen R-7 which belongs to rye grass group I allergen [12]. This structural similarity may account for the observed antigenic cross-reactivity (i.e., reactivity towards mAb 60) between *Poa p* I and *Lol p* I. The isoforms, termed as isoallergens, had been previously detected for *Lol p* I [17]. To my knowledge, this is the first report of *Poa p* isoallergens and their amino acid analysis. According to WHO allergen nomenclature criteria [18], the four isoallergens of *Poa p* I were tentatively designated as (in decreasing pI): *Poa p* IA, *Poa p* IB, *Poa p* IC and *Poa p* ID.

In view of the recent observation [2] that several clones of a cDNA library of KBG pollen allergen constructed in bacteriophage, lambda gt 11 were positive with mAb 60, the amino acid composition of the isoallergens reacting to the same mAb will also provide an invaluable aid in determining which isoform of the allergen has been cloned. No homology with the N-terminal sequence of *Poa p* I was observed with the deduced amino acid sequence from cDNA of another KBG pollen allergen [3].

The similarity in amino acid composition between the two major isoallergens of *Poa p* I indicates a high degree of homology in their amino acid sequence. A 95% sequence homology within a group of multiple clones at the protein level of a ragweed allergen *Amb a* I has recently been noted [19]. These isoallergens thus represent an excellent group of proteins to (i) study the genetic basis of allergic response [20], and (ii) also examine the epitopes recognized by T cells and by B cells.

### Acknowledgements

The technical assistance of Ms. Leila El-Kassaby is gratefully appreciated. The author wishes to thank Ms. Sandy Kielland of the University of Victoria, B.C., for carrying out the amino acid analyses and to his former colleagues [5] for their assistance in the preparation of mAb 60.

### References

- 1 Ekramoddoullah AKM, Kisil FT, Sehon AH: Isolation of a Kentucky bluegrass pollen allergen using a murine monoclonal antibody immunosorbent. *Int Arch Allergy Appl Immunol* 1986;80:100-106.
- 2 Hatton TW, Ekramoddoullah AKM, Kisil FT, Sehon AH, Hill RD: Identification of cDNA clones representing group I pollen allergens from Kentucky bluegrass; in Said El Shami A, Merrett TG (eds): *Allergy and Molecular Biology. Advances in the Biosciences*. New York, Pergamon Press, 1988, vol 74, pp 173-176.
- 3 Mohapatra SS, Hill RD, Astwood J, Ekramoddoullah AKM, Olsen E, Silvanovitch A, Hatton T, Kisil FT, Sehon AH: Isolation and characterization of a cDNA clone encoding a major allergen from Kentucky bluegrass (*Poa pratensis*) pollen. *Int Arch Allergy Appl Immunol* 1990;91:362-368.
- 4 Lin Z, Ekramoddoullah AKM, Kisil FT, Hebert J, Mourad W: Isolation and characterization of *Poa p* I allergens of Kentucky bluegrass pollen with a murine monoclonal anti-*Lol p* I antibody. *Int Arch Allergy Appl Immunol* 1988;87:294-300.
- 5 Lin Z, Ekramoddoullah AKM, Kisil FT, Jaggi KS, Dzuba-Fischer J, Rector E: Mapping of epitopes on *Poa p* I and *Lol p* I allergens with monoclonal antibodies. *Int Arch Allergy Appl Immunol* 1990;91:217-223.
- 6 Hochstrasser DF, Harrington MG, Hochstrasser A-C, Miller MJ, Merrill CR: Methods for increasing the resolution of two-dimensional protein electrophoresis. *Analyt Biochem* 1988; 173:424-435.
- 7 Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci USA* 1979;76:4350-4354.
- 8 Matsudaira P: Sequence from picomole quantities of proteins electrophoretically onto polyvinylidene difluoride membranes. *J Biol Chem* 1987;262:10035.
- 9 Cottam GP, Moran DM, Standring R: Physicochemical and immunochemical characterization of allergenic proteins from rye-grass (*Lolium perenne*). *Biochem J* 1986;234:305.
- 10 Garrels JI, Farrar JT, Burwell CB IV: The QUEST system for computer-analyzed two-dimensional electrophoresis of proteins; in Cells JE, Bravo R (eds): *Two-Dimensional Gel Electrophoresis of Proteins: Methods and Applications*. New York, Academic Press, 1984, pp 37-91.
- 11 Baer H, Anderson MC, Helm RM, Yuninger JW, Lowenstein H, Gjesing B, White W Jr, Douglass G, Phillips PR, Schumacher M, Hewitt B, Guerin BG, Charpin J, Carreira J, Lombardo M, Ekramoddoullah AKM, Kisil F, Einarsson R: The preparation and testing of the proposed international reference (IRP) Bermuda grass (*Cynodon dactylon*) pollen extract. *J Allergy Clin Immunol* 1986;78:624-631.
- 12 Stewart GA, Turner KH, Baldo BA, Cripps AW, Ford A, Seagroatt V, Lowenstein H, Ekramoddoullah AKM: Standardization of rye-grass pollen (*Lolium perenne*) extract: An immunochemical and physicochemical assessment of six candidate international reference preparations. *Int Arch Allergy Appl Immunol* 1988;86:9-18.
- 13 Chakrabarty S, Lowenstein H, Ekramoddoullah AKW, Kisil FT, Sehon AH: Detection of cross-reactive allergens in Kentucky bluegrass and six other grasses by crossed radioimmuno-

- noelectrophoresis. *Int Arch Allergy Appl Immunol* 1981;66:142-157.
- 14 Ekramoddoullah AKM, Kisil FT, Sehon AH: Allergenic cross-reactivity of cytochrome c of Kentucky bluegrass and perennial ryegrass pollens. *Mol Immunol* 1982;19:1527-1534.
- 15 Ekramoddoullah AKM, Kisil FT, Bundesen PG, Fischer JMM, Rector ES, Sehon AH: Determinants of ryegrass pollen cytochrome c recognized by human IgE and murine monoclonal antibodies. *Mol Immunol* 1982;21:375-382.
- 16 Ekramoddoullah AKM, Kisil FT, Sehon AH: Partial characterization of an antigenic site of high molecular weight basic antigen, a ryegrass pollen allergen, using a monoclonal antibody. *Mol Immunol* 1986;23:111-117.
- 17 Johnson P, Marsh DG: 'Isoallergens' from ryegrass pollen. *Nature* 1965;206:935.
- 18 Marsh DG, Goodfriend L, King TP, Lowenstein H, Platts-Mills TAE: Terminology, allergen nomenclature. *Bull World Health Organ* 1986;64:767-770.
- 19 Rafner T, Bond JF, Rogers BL, Klapper DG: Cloning of short ragweed antigen E and placement of its alpha and beta chains (abstract). *FASEB J* 1990;4:A1938.
- 20 Marsh DG, Zwollo P, Ansari AA: Towards a total immune response fingerprint: the allergy model; in Said El Shami A, Merrett TG (eds): *Allergy and Molecular Biology. Advances in the Biosciences*. New York, Pergamon Press, 1988, vol 74, pp 65-82.

Received: October 22, 1990

Accepted after revision: December 18, 1990

Correspondence to: Dr. Abul K.M. Ekramoddoullah  
Pacific Forestry Centre  
Forestry Canada  
506 West Burnside Road  
Victoria, BC V8Z 1M5 (Canada)