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Isolation of *Der pI*, the *Dermatophagoides pteronyssinus* major mite allergen, from a crude mite culture extract, purification by ion-chromatography, and comparison between the material obtained and a cDNA-coded *Der pI*

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ABSTRACT

A high degree of purity is a prerequisite for an allergen preparation to be suitable for clinical diagnosis and therapy. A pure allerge can easily be obtained from a crude mite culture extact by using an immunosorbent prepared with highly specific monoclonal antibodi or from a cDNA-coded material. However, up to now none of these methods has been performed on a process scale. Here large-sca purification is defined as a process in which a crude *Dermatophagoides pteronyssinus* mite culture extract is essentially fractionated b acetone and ammonium sulphate precipitations followed by anion-exchange high-performance liquid chromatography. A high yield a very pure *Der pI* allergen is obtained during the first isocratic run, as shown by sodium dodecylsulphate–polyacrylamide gel electr phoresis, capillary electrophoresis, chromatofocusing and a two site monoclonal antibody enzyme-linked immunosorbent assay. M crosequencing revealed that the 25-residue sequence obtained is entirely in agreement with the sequence derived from the cDNA of *D pI*.

INTRODUCTION

The major allergen from *Dermatophagoides pteronyssinus* mite was first isolated about 10 years ago [1–3] and was shown to have common epitopes with the corresponding major allergen of the *Dermatophagoides farinae* mite [4], a second species of the same arthropod family. Both allergens were isolated by several authors but *Der pI* (named p1, Ag 7 or Ag 42 before standardization [5]) was more purified in particular by Chapman and Platts-Mills [1] from *D. pteronyssinus* mite culture and *Der fI* (named Ag 11, Ag 21, Ag 6 before standardization [5]) by Dandeu *et al.* [6] from a *D. farinae* mite culture.

Both *Der pI* and *Der fI* were stated to be single peptides with some traces of carbohydrates. The binding of these to the protein moiety was not full demonstrated. Similarity between its inferred ami no acid sequence and the group of cysteine proteas es appeared from the sequence of the mRNA [7,8 and secondly of the cDNA [9] coding for *Der p* Here we confirm these observations, as our result essentially do not differ from those of the above mentioned experiments, at least for the amino aci sequence of the 25-residue N-terminal peptide c *Der pI*, biochemically prepared from a crude mit culture extract.

EXPERIMENTAL

Dp 80d

A partially purified extract was prepared fror

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the crude mite culture extract obtained according to the method described previously in detail [10] by exhaustive dialysis against water [6]. When the absorbance of the dialysate reached zero, the resulting brownish solution was named Dp 80d.

The A 60 fraction

A very enriched *Der pI* fraction was obtained at 60% ammonium sulphate saturation as described previously [6] for *D. farinae*. The precipitate was dissolved in water, exhaustively dialysed against water and lyophilized.

Anion-exchange chromatography

Preliminary experiments showed that all of the allergenic components of A 60 were negatively charged [4], so an anion exchanger was used to separate them. A Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden) controlled by a fast protein liquid chromatographic (FPLC) system (Pharmacia) was equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.60).

A 200-mg sample of A 60 dissolved in the same buffer was loaded on to the column. Stepwise elution was performed: first an isocratic run with the equilibration buffer was carried out at 1 ml/min followed by a second and third steps with the same buffer containing 1 *M* and 2 *M* NaCl, respectively, at 3 ml/min. The absorbance was monitored at 280 nm. Thereafter the column was re-equilibrated and used again.

Two-site monoclonal antibody enzyme-linked immunosorbent assay (Mab ELISA)

The identity and degree of purity of the *Der pI* allergen obtained by the process described above were controlled by a two-site Mab ELISA using anti-*Der pI* (5H8) monoclonal antibodies and anti-*Der pI* (4C1) biotinylated monoclonal antibodies from Charlottesville University, VA, USA), Martin Chapman Laboratory, according to the method described by Luczynska *et al.* [11].

A CEB immunoplate (Centre Européen de Biotechnologie, France) was coated with 1 μg per well of Mab 5H8 (anti-*Der pI*, 10 mg/ml) in 0.1 *M* sodium hydrogencarbonate buffer (pH 9.6) overnight at 4°C. The plate was then washed twice with phosphate-buffered saline (PBS) containing 1% of Tween 20 (pH 7.4) and treated for 1 h with 100 μl per well of the same solution containing 1% of bovine serum albumine.

The wells were incubated for 1 h at room temperature with 100 μl of diluted fractions (from 10^{-2} to 10^{-7}) using double dilutions of a reference antigen, *Der pI* International Standard Dp 82518, to make a calibration graph (from 250 to 3.9 ng).

After washing five times, the wells were incubated for 1 h at room temperature with 100 μl of a 1:1000 dilution of biotinylated Mab 4C1 (anti-*Der pI*, 10 mg/ml). The plate was then washed five more times and the wells were incubated for 30 min with 1:1000 streptavidin–peroxidase (Sigma, St. Louis, MO, USA) (0.25 mg of protein was dissolved in 1 ml of distilled water). Finally, the assays were developed by adding 100 μl per well of 1 m*M* ABTS [2,2′-azino di(3-ethylbenzthiazoline-6-sulphonic acid)] and 5% hydrogen peroxide in 70 m*M* citrate–phosphate buffer (pH 4.2) (Sigma). The reaction was stopped after 10 min by adding 100 μl per well of 2 m*M* sodium azide solution. Absorbance was monitored at 414 nm in an ELISA microplate reader.

Capillary electrophoresis

This was performed in an HPE TM 100 system (Bio-Rad, France) at 8 kV/cm for 8 s on a coated HPE capillary cartridge (50 cm × 50 μm I.D.), from Bio-Rad. A 0.1 *M* phosphate buffer (pH 2.5) from Bio-Rad was used. Absorbance was monitored at 214 nm.

Chromatofocusing

A Mono P column of the HR 5/20 type (Pharmacia) was used after being equilibrated with 0.025 *M* Tris buffer (pH 8). The protein of interest was dissolved in the same buffer and loaded on to the column. A linear concentration gradient was applied, between 0 and 100% of Servalytes 2–4 at 40% (Serva, Heidelberg, Germany) diluted with water to 0.2% and adjusted to pH 3 with HCl. The pH gradient was recorded using a pH monitor (Pharmacia). Absorbance was monitored at 280 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Analyses were performed using homogeneous 15% gel on a Hoefner apparatus. Before electrophoresis samples were treated with SDS or with SDS and dithiotreitol (50 m*M*) as reducer. After electrophoresis Coomassie Brilliant Blue staining was used.

Amino acid analysis

The amino acid compositions were determined with a Beckman Model 7300 amino acid analyser after acidic hydrolysis performed in the vapour phase with 6 *M* HCl containing 0.1% of phenol for 24 h at 110°C *in vacuo*, according to Moore and Stein [12].

Reversed-phase high-performance liquid chromatography (RP-HPLC)

RP-HPLC purification of the allergenic fraction was performed on a Brownlee Labs. Aquapore butyl column (220 mm × 2.1 mm I.D.), using a linear gradient of acetonitrile in water, containing 0.1% of TFA, from 31 to 50% acetonitrile in 60 min at 200 μl/min. The process was controlled by an HPLC system (Pharmacia–LKB). Absorption was monitored at 214 nm.

Protein sequencing and amino acid sequence homology determination

Proteins were sequenced on a Model 470 gas-phase protein sequencer (applied Biosystems, France). Phenylthiohydantoin (PTH) derivatives of amino acids were separated and identified by on-line RP-HPLC with an RP-18 column (Brownlee Labs). Biobrene (Applied Biosystems) was added to prevent wash-out and to improve the initial yields [13].

RESULTS

Dp 80d

Both *D. pteronyssinus* and *D. farinae* mite culture were generally performed on a human danders and yeast extract-supplemented medium. Extraction with an aqueous medium and acetone treatment were first described by Guibert and Causse-Combes [10] and led to a material we named Dp 80 (or Df 80), which was exhaustively dialysed against distilled water in order to eliminate the low-molecular mass molecules, and named "partially purified extract" Dp 80d or Df 80d [4].

Dp A 60

A major allergen-enriched fraction, either *Der pI* or *Der fI* was obtained by ammonium sulphate p cipitation at 60% saturation, as was previously scribed for *Der fI* [6]. Dp A 60 is a very enriched *I pI* fraction, as shown by two-site Mab ELIS which indicated a concentration of at least 78% *Der pI* when compared with the International St dard, as a solution at 2.3 mg/ml contains 1.8 mg/ of *Der pI*. Therefore, further purification was nec sary and the following process was applied.

Anion-exchange chromatography

Using anion-exchange chromatography perfor ed on microbeads of a strong anion exchang Mono Q, we were able to purify *Der pI* in a sin step. A 200-mg sample of the Dp A 60 fraction w dissolved in and dialysed against solution A, 0.02 Tris–HCl buffer (pH 8.6), and loaded on to t Mono Q HR 10/10 column which had previou been equilibrated with the same buffer.

During the first, isocratic run, with solution only one peak of non-adsorbed material was elut The material strongly bound to the support w desorbed by adding solution B diluted 1:2 with s lution A [solution B = 0.02 *M* HCl buffer (pH 8 containing 2 *M* NaCl]. Finally, in order to deso more strongly bound material essentially compos of brownish pigments, the column was washed wi 100% solution B.

The elution profile obtained is shown in Fig. 1

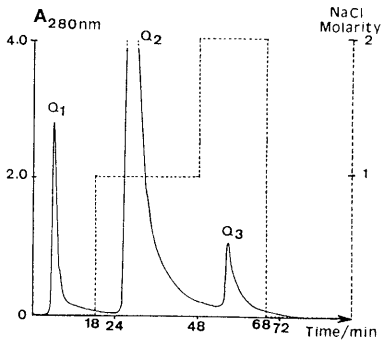

Fig. 1. Chromatogram of Dp 80d on a Mono Q HR 10/10 c umn equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.6) (soluti A). Elution was carried out with 1 *M* NaCl in 0.02 *M* Tris–H buffer (pH 8.6) (50% solution B), flow-rate 1 ml/min, follow by 2 *M* NaCl in 0.02 *M* NaCl in 0.02 *M* Tris–HCl buffer (pH 8 (100% solution B). Flow-rate, 3 ml/min. UV detection at 2 nm.

The three fractions obtained by this procedure were named Q1, Q2 and Q3 and were tested for their *Der pI* content by the two-site Mab ELISA. Only Q1 contained a significant amount of *Der pI*, *i.e.*, 100%.

The Q1 fraction was analysed using SDS-PAGE, both before and after a reduction treatment with dithiothreitol. In the former instance three bands were observed, with molecular masses (M_r) 25 000, 15 500 and 14 500. In the latter instance only two bands were observed, with M_r 15 000 and 13 500 (Fig. 2).

Only one major peak was found to occur in capillary electrophoresis (Fig. 3). Chromatofocusing (Fig. 4) indicated a p*I* of *ca.* 7.85. Both of these experiments indicated a great homogeneity for the Q1 fraction.

Table I shows the amino acid composition of Q1 in comparison with that found for a cDNA-coded *Der pI* prepared by other workers [7–9]. Significant differences appear for some amino acid residues: in our *Der pI* preparation there is twice as much proline whereas isoleucine, histidine and arginine are present at a concentration half of those determined for the cDNA preparation. There is four times less tyrosine and about five times more lysine.

Der pI purified as described above was submitted to Edman degradation for N-terminal sequence analysis. However, owing to the high background,

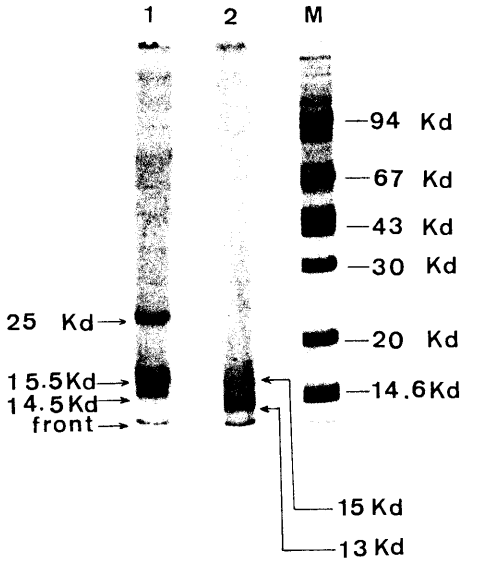

Fig. 2. SDS-PAGE performed in a homogeneous 15% gel. A 1-μl sample of either dithiothreitol-treated or untreated Q1 fraction, corresponding to 10 μg dry weight, was deposited. The gel was stained with Coomassie Brilliant Blue. M = protein markers of known molecular mass. 1 = Native *Der pI* (Q1); 2 = *Der pI* (Q1) treated with dithiothreitol. Kd = kilodaltons.

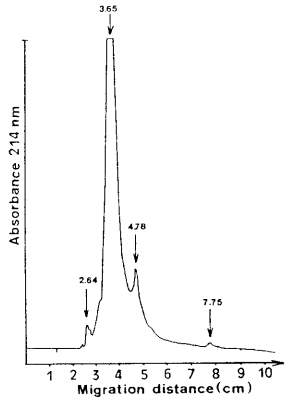

Fig. 3. Electropherogram obtained by capillary electrophoresis as described in the text.

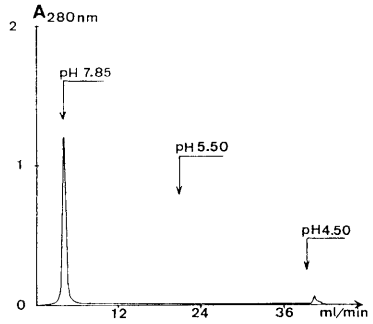

Fig. 4. Chromatofocusing with a Mono P HR 5/20 column equilibrated with 0.025 *M* Tris–HCl buffer (pH 8.00). An 8.5-mg amount of *Der pI* (Q1) was loaded on the column. Elution was performed at a flow-rate of 1.0 ml/min with a Servalytes 2–4 solution at 0.2% adjusted to pH 3.00. The pH gradient was recorded using a pH monitor.

TABLE I

AMINO ACID COMPOSITION

Amino acid	Theoretical (mol%)[a]	*Der pI* (mol%)
Asx	13.4	9.5
Thr	3.7	5.3
Ser	5.1	6.8
Glx	11.6	18.0
Pro	4.2	9.8
Gly	7.8	8.9
Ala	9.2	8.1
Cys	3.7	3.4
Val	5.5	5.4
Ile	7.8	3.6
Leu	4.2	5.6
Met	1.8	1.1
Tyr	8.3	2.0
Phe	1.8	2.2
His	3.2	1.9
Lys	0.9	4.6
Arg	7.8	3.8
Total	100.0	100.0
Trp	—[b]	—[b]

[a] From the cDNA sequence [8].
[b] Not determined.

the first residues were not identified. To overcome this problem, the Q1 fraction was further purified by RP-HPLC to remove contaminating products unrevealed by other analyses. Their presence, however, seems not to influence the allergenic and antigenic activity. The chromatographic data (Fig. 5) showed a sharp peak of non-adsorbed material, only detectable at 214 nm, and major broad overlapping peaks which were collected individually, denoted A, B, C, D and E, respectively and reseparated to homogeneity. Several of these peaks were sequenced for N-terminal analysis. This failed to show differences among the N-terminal residues, suggesting heterogeneity elsewhere in the molecule.

The common 25-residue sequence obtained is shown in Table II. An extent of 52% of homology were found with two cysteine proteases and almost 100% with the cDNA-coded *Der pI* (Table II).

DISCUSSION

Advances in biotechnology have permitted the synthesis and recovery of the specific DNA coding for a given protein and the cloning of it in certain

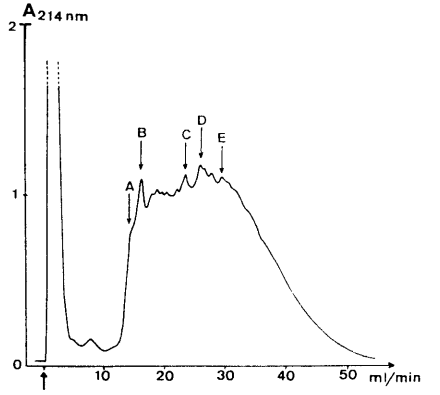

Fig. 5. RP-HPLC purification of the allergenic fraction Q1 formed on an Aquapore butyl column (220 mm × 2.1 mm I as described in the text.

microorganisms. Unfortunately, the protein of terest, although the majority component, is p duced together with natural proteins from whic has to be isolated.

A cDNA clone coding for the major house d *D. pteronyssinus* mite allergen *Der pI* was isola from a lambda gt 11 library [14]. It produces a sion protein which reacts with rabbit immunoglobulin G (IgG) antibodies raised against *Der pI* does not react with human IgE antibodies pres in sera from mite-sensitized patients, or at least o very weakly [15]. Moreover, the amount of solu fusion protein in the bacterial lysate is very low a so far the biochemical preparation of a pure a highly allergenic *Der pI* from mite culture extr remains the only scale process available.

Either from a bacterial lysate or from a mite c ture extract, separation and purification of suc biopolymer is performed using liquid chromatog phy.

Because no two proteins are alike, meth which give satisfactory results with some prote cannot be used with others. Nevertheless, anion cathion exchangers are generally effective with m proteins. This was illustrated by the purificatior two other important allergens. The cat major lergen, *Fel dI* was isolated and purified from a ho dust extract, a very complex mixture of organic a

TABLE II

Der pI HOMOLOGIES (N-TERMINUS): N-TERMINUS COMPARISON

Cysteinyl protease (23)						I	N	G	K	A	T	A	L	A	N	L	R	K	S	R					
						:	:	:		:		:				:	:			:					
cDNA-coded *Der pI* (8)	T	N	A	C	S	I	N	G	N	A	P	A	E	I	D	L	R	Q	M	R	T	V	T	P	I
	:	:	:		:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
Biochemicaly purified *Der pI*	T	N	A	?	S	I	N	G	N	A	P	A	E	I	D	L	R	Q	M	R	T	V	T	P	I
											:			:	:		:					:	:	:	:
Cysteine proteinase 2 precursor (22)											P	K	S	I	D	W	R	T	K	N	A	V	T	P	I

inorganic molecules, using anion-exchange chromatography followed by copper chelate chromatography [16]. Cat serum albumin (CSA), a very potent allergen, was isolated and purified using a similar procedure applied to a cat sera pool [17].

Here we have reported the large-scale preparation of a highly pure *Der pI* using a very simple chromatographic process. This process essentially consists of anion-exchange chromatography on a Mono Q column applied to an ammonium sulphate fraction obtained from a partially purified mite culture extract. The protein of interest is eluted during the first isocratic part of the run. All the contaminants remain strongly bound to the support.

We used an original two-site monoclonal antibody assay described by Luczynska *et al.* [11] to evaluate the concentration and allergenic activity of the *Der pI* allergen, whatever its origin. Monoclonal antibodies significantly improved the standardization of most clinically relevant allergens.

Several techniques for determining the composition and activity of allergens are available. This essentially depends on the specific IgE-containing human sera pool or on the patient populations tested, *e.g.*, radio-allergo-sorbent test (RAST) or crossed radio-immunoelectrophoresis (CRIE). With monoclonal antibodies, as a consequence of their high specificity and homogeneity, a very effective method has been developed in which epitope recognition is the main factor in allergen identification. A prerequisite is the identity between epitopes recognized by human IgE antibodies and these monoclonal antibodies. This has been particularly well reviewed by Diener and Jäger [18]. Two-site immunoassays seem to be the method of choice. Hence we used this assay in comparison with an international standard of the allergen studied and the corresponding monoclonal antibodies [11].

This approach allowed a high degree of allergenic activity for the *Der pI* allergen we isolated and purified to be demonstrated. Moreover, this was confirmed using a histamine release experiment where this pure allergen was tested on basophilic cells from mite-sensitized human patients (results not shown) [19].

In order to assess the real homogeneity of this *Der pI* preparation we performed SDS-PAGE. Here the major component had $M_r \approx 25\,000$, a result very close to that reported by other workers [1,8]. Two other components with M_r 15 500 and 14 500 are also present and probably result from denaturation or degradation. These two lower-M_r peptides can be obtained from the major component by a reducing treatment of the S–S bonds.

These results lead to the suggestion that *Der pI* could consist of two peptides probably held together by one or several disulphide bridges. *Der pI* in the Q1 fraction appears highly homogeneous in capillary electrophoresis, the most critical method of analysis currently available. The relative heterogeneity observed in RP-HPLC may reflect C-terminal variations of the protein (length or amino acid content, a conclusion supported by the results of the amino acid analysis), but may also result from glycosylation of *Der pI*. Although a potential glycosylation site is present in its sequence, no significant amount of saccharides has been found. On chromatofocusing it also appears homogeneous and has a $pI \approx 7.85$, which differs from the $pI \approx 6.6$ mentioned by other workers [1], but this may be explained by the above remarks.

On the other hand, at the level of the 1–25 N-terminal peptide sequence no differences appear when compared with the same peptide of the cDNA-coded *Der pI*.

As noted above, the cDNA-coded *Der pI* only reacts with rabbit IgG antibodies while its allergenicity was shown to be poor when tested with human specific IgE [15]. The same authors [20] isolated a cDNA coding for the second major allergen from *D. pteronyssinus* mite, *Der pII*, which expressed a high allergenic activity. It should also be noted that Tovey *et al.* [21] cloned and sequenced a cDNA-coded recombinant house dust mite protein that binds human IgE, but it was not identified.

The allergenicity of *Der pI* could be highly dependent on its tertiary structure, which is probably not very well conserved in the cloning process. Post-translational modifications constitute an unpredictable group of changes that can lead to a post-synthetic active or inactive protein. The nature of these changes can be determined by protein sequencing without anticipating their influence on the secondary and tertiary structures. Tertiary structure has the most important function both in the antigenicity and in the allergenicity of a protein. It is also well known that a change of only one amino acid residue in the active site of an enzyme can have a dramatic effect [22]. It should be noted that the catalytic site is particularly conserved in cysteine proteases of highly divergent organisms [23] and this is very important when considering amino acid sequence homology, which has been recognized for *Der pI* with several cysteine proteases. A 25% homology was noted on comparing the sequence of the *Der pI* cDNA clone and its inferred amino acid sequence with those of proteins already analysed [8]. We confirmed this homology on the basis of the 1–25 N-terminal peptide sequence, *i.e.*, 52% homology with the cysteine proteinase 2 precursor (E.C. 7.4.22–.) from *Dictyostelium discoideum* (slime mould) [24] and the cysteinyl protease from *Asclepia syriaca* L. (milkweed) (E.C. 3.4.22.7) [25]. These results do not allow any conclusions to be drawn concerning the role or the origin of the *Der pI* protein.

The availability of large amounts of pure allergen will allow further studies of these points and also of hypersensitization, hyposensitization or tolerance induction in animals for a better understanding of these phenomena in humans.

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REFERENCES

1 M. D. Chapman and T. A. E. Platts-Mills, *J. Immunol.*, 1 (1980) 87.
2 S. Krilis, B. A. Baldo and A. Basten, *J. Allergy Clin. Immunol.*, 74 (1984) 142.
3 P. Lind and H. Löwenstein, *Scand. J. Immunol.*, 17 (198 263.
4 J. Le Mao, J.-P. Dandeu, J. Rabillon, M. Lux and B. Davi *J. Allergy Clin. Immunol.*, 71 (1983) 588.
5 D. G. Marsh, *Bull. WHO*, 64 (1986) 767.
6 J. P. Dandeu, J. Le Mao, M. Lux, J. Rabillon and B. Davi *Immunology*, 46 (1982) 107.
7 G. A. Stewart and W. R. Thomas, *Int. Arch. Allergy Ap Immunol.*, 83 (1987) 384.
8 K. Y. Chua, G. A. Stewart, W. R. Thomas, R. J. Simpson, J. Dilworth, T. M. Plozza and K. J. Turner, *J. Exp. Med.*, 1 (1988) 175.
9 G. A. Stewart, W. R. Thomson, K. Y. Chua, and H. M Geysen, *Adv. Biosci.*, 74 (1989) 297.
10 L. Guibert and R. Causse-Combes, *Ann. Inst. Pasteur*, 1 (1965) 579.
11 C. M. Luczynska, L. K. Arruda, T. A. E. Platts-Mills, J. Miller, M. Lopez and M. D. Chapman, *J. Immunol. Metho* 118 (1989) 227.
12 S. Moore and W. H. Stein, *J. Biol. Chem.*, 176 (1948) 367
13 G. E. Tarr, J. F. Beecher, M. Bell and D. McKean, *An Biochem.*, 84 (1978) 622.
14 T. V. Huynh, R. A. Young and R. W. Davis, in D. M. Glov (Editor), *DNA Cloning. Vol. 1, a Practical Approach*, IR Press, Oxford, Washington, 1985, pp. 48–78.
15 W. R. Thomas, G. A. Stewart, R. J. Simpson, K. Y. Chua, M. Plozza, R. J. Dilworth, A. Nisbet and K. J. Turner, *In Arch. Allergy Appl. Immunol.*, 85 (1988) 127.
16 J.-P. Dandeu, J. Rabillon, M.-J. Beltrand, M. Lux, R. Duv and B. David, *J. Chromatogr.*, 512 (1990) 177.
17 J.-P. Dandeu, J. Rabillon, J.-L. Guillaume, L. Camoin, M Lux and B. David, *J. Chromatogr.*, 539 (1991) 475.
18 C. Diener and L. Jäger, *Res. Trends, ACI News*, 1 No. (1989) 180.
19 A. Weyer, personal communication.
20 K. Y. Chua, C. R. Doyle, R. J. Simpson, K. J. Turner, G. A Stewart and W. R. Thomas, *Int. Arch. Allergy Appl. Immu nol.* 91 (1990) 118.
21 E. R. Tovey, M. C. Johnson, A. L. Roche, G. S. Cobon an B. A. Baldo, *J. Exp. Med.*, 170 (1989) 1457.
22 J. F. Bazan and R. J. Fletterick, *Proc. Natl. Acad. Sc U.S.A.*, 85 (1988) 7872.
23 A. N. Eakin, J. N. Higaki, J. H. McKerrow and C. S. Craik *Nature (London)*, 342 (1989) 132.
24 C. J. Pears, H. M. Mahbuni and J. G. Williams, *Nucle Acids Res.*, 13 (1985) 8853.
25 L. A. Allison, M. Moyle, M. Shales and C. J. Ingles, *Cell*, 4 (1985) 599.