

# Members of the $\alpha$ -amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma

Luis Gómez, Elena Martín\*, Dolores Hernández\*, Rosa Sánchez-Monge, Domingo Barber<sup>+</sup>, Victoria del Pozo\*, Belén de Andrés\*, Alicia Armentia<sup>o</sup>, Carlos Lahoz\*, Gabriel Salcedo and Pilar Palomino\*

*Departamento de Bioquímica, ETS Ingenieros Agrónomos, Madrid, \*Departamento de Inmunología, Fundación Jimenez Diaz, Madrid, <sup>+</sup>Departamento de Análisis, Alergia e Inmunología Abelló, Madrid and <sup>o</sup>Sección de Alergia, Hospital Rio Ortega, Valladolid, Spain*

Received 27 November 1989

We have identified the major antigens or IgE binding components from wheat flour. Thirty-five sera from patients with baker's asthma were used to analyze the reaction with wheat salt-soluble proteins. We found a 15 kDa SDS-PAGE band which reacted with all sera tested. Purified members of the  $\alpha$ -amylase inhibitor family, which are the main components of the 15 kDa band, were recognized by specific IgE when tested with a pool of reactive sera. Immunodetection after two-dimensional electrophoretic fractionation of crude inhibitor preparations from wheat endosperms also detected several inhibitor subunits as major low-molecular-weight allergens.

Allergen; Baker's asthma;  $\alpha$ -Amylase inhibitor; (wheat)

## 1. INTRODUCTION

Baker's asthma due to wheat allergens is an occupational disease with elevated prevalence [1-3]. Although a certain degree of cross-reactivity has been described among antigens from different cereal species [4-6], it is well established that the wheat antigens are the most reactive allergens [2,5,7,8]. The most prominent allergens in wheat flour have been related to the salt-soluble fraction [9,10], but little progress has been made in their isolation and characterization [6,11]. Walsh and Howden [12], using an epitope mapping method, have recently identified a putative allergenic peptide in the NH<sub>2</sub>-terminal amino acid sequence of protein 0.28, a wheat monomeric inhibitor of heterologous  $\alpha$ -amylases. A major allergen with activity against insect  $\alpha$ -amylase has been also characterized in barley flour [13]. Both proteins have around 14 kDa, and belong to the same protein family in cereals, which includes trypsin inhibitors and subunits of monomeric, dimeric and tetrameric inhibitors of heterologous  $\alpha$ -amylases [14,15].

*Correspondence address:* P. Palomino, Depto. Inmunología, Fundación Jimenez Diaz, Av. Reyes Católicos 2, 28040 Madrid, Spain

*Abbreviations:* IEF, isoelectrofocusing; PVDF, polyvinylidene difluoride; RAST, radioallergosorbent test; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SGE, starch gel electrophoresis; WDAI-3, wheat dimeric  $\alpha$ -amylase inhibitor-3

We have now investigated the IgE binding capacity of salt-soluble proteins from wheat flour using 35 sera from bakers with symptoms of rhinoconjunctivitis and/or asthma due to inhalation of wheat flour. We also report the identification of members of the  $\alpha$ -amylase inhibitors family, present both in hexaploid (bread) and tetraploid (pasta) wheats, as the main low-molecular-weight allergens associated with baker's asthma.

## 2. MATERIALS AND METHODS

Thirty-five sera were obtained from blood samples of patients with baker's asthma. The sera were RAST class 4 when assayed with wheat flour allergens from Pharmacia (Pharmacia Diagnostics, Uppsala, Sweden). As control we used sera from healthy donors. All sera were stored at -80°C until used.

Salt-soluble proteins from bread wheat flour (0.15 M NaCl extract) and crude inhibitor preparations from *T. aestivum* (cv. Chinese Spring) and *T. turgidum* (cv. Senatore Capelli) endosperms were obtained essentially as in [16]. Purification of  $\alpha$ -amylase inhibitors subunits was performed by gel filtration on Sephadex G-100 followed by reversed-phase high-performance liquid chromatography or ion-exchange chromatography [15,17,18]. Protein concentration was determined by standard methods [19,20].

SDS-PAGE was carried out according to Laemmli [21] and electroblotting of proteins on nitrocellulose or PVDF membranes (Immobilon, pore size 0.45  $\mu$ M, Millipore) was essentially as in [22]. Two-dimensional electrophoresis by combined IEF  $\times$  SGE was as described [23]. Before electroblotting, each 2 mm thick starch-gel was sliced in two slabs replicas (1 mm thick) that were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol), for 40 min. PVDF membranes were soaked in 100% methanol and stored in transfer buffer. Each 1 mm slab replica was

sandwiched between a sheet of PVDF membrane and several sheets of Whatman paper 3MM, placed into a semi-dry transfer unit (Semi-Phor TE-70, Hoefer Sci. Inst., San Francisco, CA), and electrotransferred at 1 mA/cm<sup>2</sup> for 1 h with external cooling. Coomassie blue staining of PVDF membranes was as in [24].

For immunodetection [25], after treatment of membranes with appropriate sera dilutions, rabbit anti-human IgE and IgG conjugated with  $\beta$ -galactosidase (Pharmacia) were used. Substrate solution was  $\beta$ -naftyl-galactopiranoside. Specific IgE and IgG were determined by Pharmacia  $\beta$ -galactosidase Phadezym RAST.

### 3. RESULTS AND DISCUSSION

A 15 kDa antigenic band from the salt-soluble proteins from wheat flour, reacted with all the 35 sera from patients with baker's asthma tested by immunodetection (fig.1), although different patterns were obtained regarding the number, molecular weight and intensity of the bands recognized by each individual serum (fig.1A). Prominent bands of 60 and 30 kDa reacted with 66% and 40% of the patient's sera, respectively. Using the same method, Pfeil et al. [26] have detected bands of 15–17 and 47 kDa.

When immunodetection was carried out with anti-gamma chain antibody, only 81% of the same sera reacted with the 15 kDa antigen and 62% with the 60 kDa one. No correlation was found between the presence of these antibodies with the allergic symptomatology or exposition time (data not shown). Lee et al. [27] have suggested that the demonstration of specific serum IgG against wheat flour antigens was a good parameter of exposition but was poorly correlated with the allergic disease.

According to the result summarized in fig.1, the 15 kDa SDS-PAGE band included the major wheat allergens. It has been shown that the main salt-soluble proteins from wheat flour with molecular mass around 13–15 kDa, are members of a family of inhibitors of heterologous  $\alpha$ -amylases [14,28]. We have purified

most members of this protein family (fig.2A). Based on amino acid sequence similarity and genetic data, the subunits of wheat  $\alpha$ -amylase inhibitors have been grouped into 5 different subfamilies, each one associated with a set of homologous loci [14,15]. These subfamilies are represented by CM1/CM2, CM3, and CM16/CM17, which are subunits of tetrameric inhibitors; 0.19/0.53, that generate homodimeric inhibitors, and 0.28, the single monomeric inhibitor characterized so far. When the isolated proteins were tested by immunodetection using a pool of positive sera (fig.2B), all of them, except CM1, were recognized by specific IgE. The different components, tested at the same protein concentration and with our pooled sera, showed distinctive responses, from a weak one in the case of CM3 to the strong ones of CM2, 0.19 and 0.53. In general, members of the same subfamily gave similar responses with the exception of protein CM2, which showed strong reactivity while its close homologue CM1 did not react. This markedly different reactivity might be due either to minor sequence differences or to different sensitivity to the fractionation procedures.

We have developed a method of protein electroblotting from starch gels, in order to analyze the IgE binding capacity of the components in crude inhibitor preparations from wheat flour. This method allowed one to obtain true replicas of the same two-dimensional (IEF  $\times$  SGE) protein map, which facilitates the identification of potential allergens in complex mixtures. Results obtained with this method for crude inhibitor preparations from bread and durum wheat flours tested with the same pool of sera used in fig.2, are presented in fig.3. These results partially confirm those obtained with the purified proteins, but some differences were found. However, the conditions used in both cases were quite different (i.e. protein amounts, reducing agents, etc.), which could explain those divergences. It has been

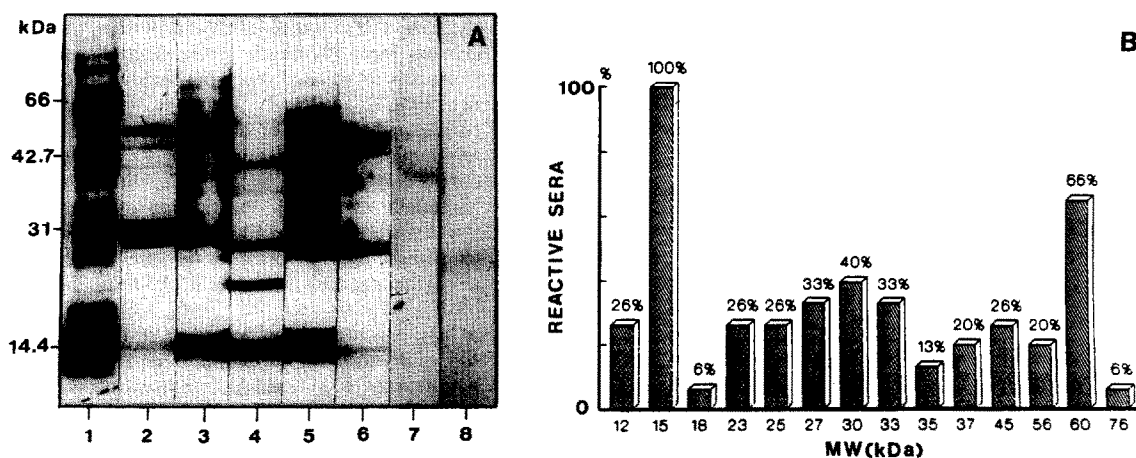


Fig.1.(A) Western blots of salt-soluble proteins (0.15 M NaCl extract) from bread wheat flour fractionated by SDS-PAGE and treated with: (1) Coomassie blue; (2–6) individual sera of patients with baker's asthma. Negative controls: (7) serum from healthy donor, and (8) serum from allergic patient with unrelated sensitization. Tracks (2–8) were exposed to anti-IgE conjugated with  $\beta$ -galactosidase. (B) Percentage of sera from 35 allergic bakers that reacted with each SDS-PAGE band.

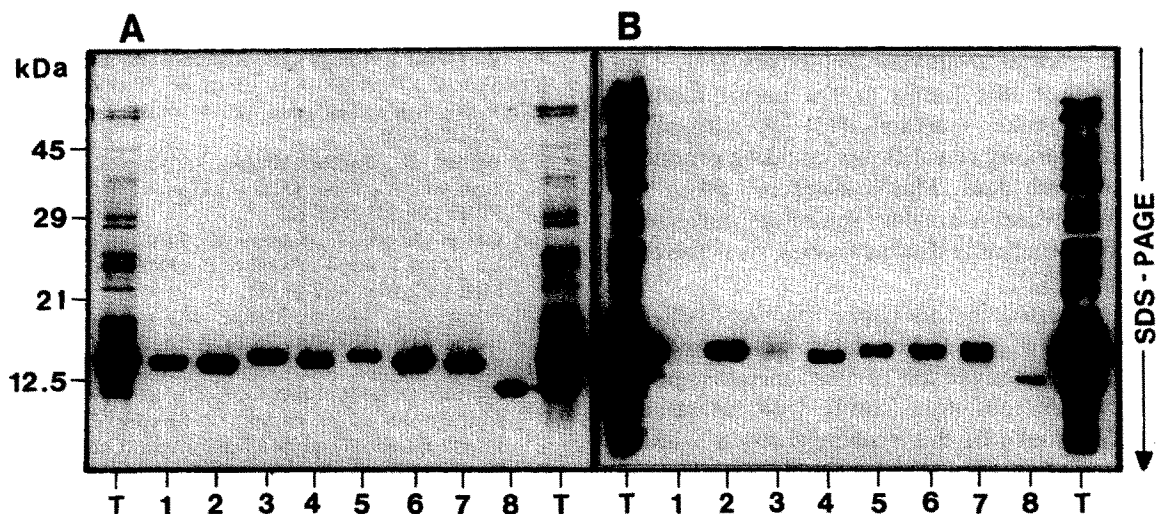


Fig.2.(A) SDS-PAGE of the following samples: (T) crude inhibitor preparation from bread wheat; purified  $\alpha$ -amylase inhibitors subunits CM1 (1), CM2 (2), CM3 (3), CM16 (4), CM17 (5), 0.19 (6), 0.53 (7) and 0.28 (8). (B) Immunoblot of a replica of the gel in A probed with pooled sera from baker's asthma patients and anti-IgE conjugated with  $\beta$ -galactosidase.

reported that variations in the fractionation and/or immunodetection methods lead to detection of different epitopes [30,31]. It should be pointed out that WDAI-3, a homodimeric inhibitor [18] was reactive in tetraploid but not in hexaploid wheat preparations. This could reflect either changes in the relative amounts of this particular component present in the two species or hidden structural differences. One of the strongest reacting components in the two-dimensional maps was an un-

characterized protein (designated UP in fig.3B,D), whose isolation and characterization is currently in progress to ascertain its possible relationship with the  $\alpha$ -amylase inhibitors family. It must be mentioned that when we used individual sera not included in the pool, different recognition patterns to those presented in fig.3 were obtained (data not shown), which is in agreement with the results summarized in fig.1.

It is known that proteins from other cereal species,

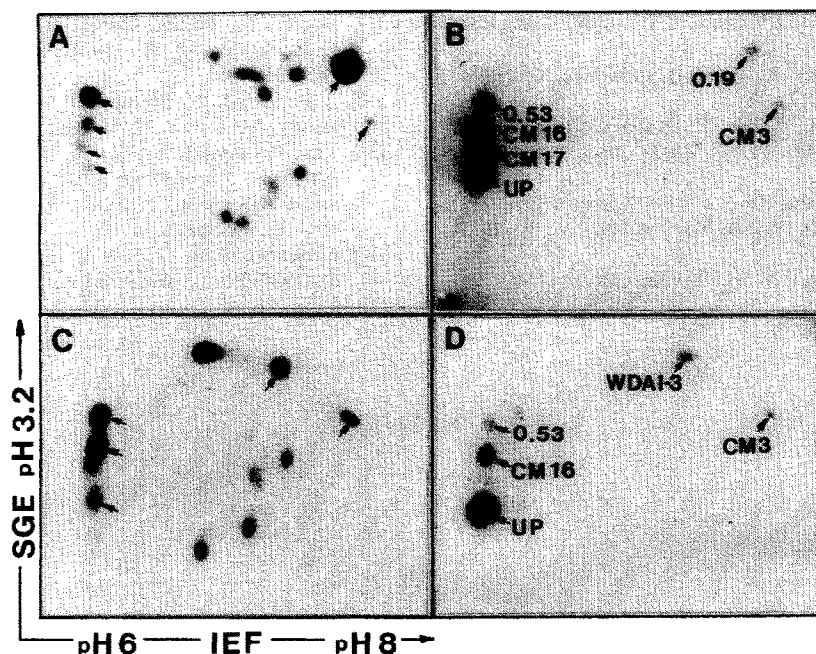


Fig.3. Electroblobs of two-dimensional electrophoretic maps (IEF, pH 6-8  $\times$  SGE, pH 3.2) of crude inhibitor preparations from bread (A,B) and durum wheat (C,D). (A,C) Stained with Coomassie blue. (B,D) Probed with pooled sera from baker's asthma patients and anti-IgE conjugated with  $\beta$ -galactosidase. Reactive  $\alpha$ -amylase inhibitors subunits are indicated, UP, uncharacterized protein. Subunits encoded by the D genome (CM1, CM17, 0.19, 0.28) are absent in durum wheat (*T. turgidum*, genomes AABB), but present in bread wheat (*T. aestivum*, genomes AABBDD).

such as barley, rye and corn, belong to the same protein family of the wheat  $\alpha$ -amylase inhibitors [14]. One barley protein of this family is the major allergen associated with baker's asthma [13]. A cross-sensitization allergy among cereal flours could be produced according to these data. Major allergens, which are homologous to wheat  $\alpha$ -amylase inhibitors, have been found in phylogenetically distant species, as is the case of *Sinapis alba* [32].

The data reported in this paper together with the epitope mapping of the 0.28 inhibitor [12], and the amino acid sequences available of many members of the  $\alpha$ -amylase/trypsin inhibitors family from cereal endosperms [14], make of this protein group a suitable model system for the study of allergic diseases associated with cereal flour manipulation.

**Acknowledgements:** We thank Dr A. Sastre and Dr E. Alvarez Cuesta (Allergy Dept., Fundación Jiménez Díaz) for clinical study of patients, Dr F. Garcia-Olmedo for critical reading of the manuscript, J. Garcia-Guijarro and P. Tramón for technical assistance. Financial support was by grants from Comisión Interministerial de Ciencia y Tecnología (No. AL 89/0121) and Fondo de Investigaciones Sanitarias de la Seguridad Social (No. 90/0238).

## REFERENCES

- [1] Popa, V., George, S.A. and Gavanescu, O. (1970) *Acta Allergol.* 25, 159-177.
- [2] Thiel, H. and Ulmer, W.T. (1980) *Chest* 78, 400-405.
- [3] Thiel, H. and Kallweit, C. (1984) *Allergologie* 7, 407-414.
- [4] Baldo, B.A., Krilis, S. and Wrigley, C.W. (1980) *Allergy* 35, 45-56.
- [5] Block, G., Tse, K.S., Kijek, K., Chan, H. and Chang-Yeung, M. (1984) *Clin. Allergy* 14, 177-185.
- [6] Blands, J., Diamant, B., Kallós, P., Kallós-Deffner, L. and Lowenstein, H. (1976) *Int. Arch. Allergy Appl. Immun.* 52, 392-406.
- [7] Granel, C., Cistero, A. and Olivé, A. (1985) *Alergia* 32, 69-73.
- [8] Prichard, M.G., Ryan, G., Walsh, B.J. and Musk, A.W. (1985) *Clin. Allergy* 15, 203-210.
- [9] Baldo, B.A. and Wrigley, C.W. (1978) *Clin. Allergy* 8, 109-124.
- [10] Theobald, K., Thiel, H., Kallweit, C., Ulmer, W. and König, W. (1986) *J. Allergy Clin. Immunol.* 78, 470-477.
- [11] Skeritt, J.H. (1988) *Adv. Cereal Sci. Technol.* 9, 263-338.
- [12] Walsh, B.J. and Howden, M.E.H. (1989) *J. Immunol. Methods* 121, 275-280.
- [13] Barber, D., Sánchez-Monge, R., Gómez, L., Carpizo, J., Armentia, A., López-Otín, C., Juan, F. and Salcedo, G. (1989) *FEBS Lett* 248, 119-122.
- [14] García-Olmedo, F., Salcedo, G., Sánchez-Monge, R., Gómez, L., Royo, J. and Carbonero, P. (1987) *Oxford Surveys Plant Mol. Cell. Biol.* 4, 275-334.
- [15] Gómez, L., Sánchez-Monge, R., García-Olmedo, F. and Salcedo, G. (1989) *Proc. Natl. Acad. Sci. USA* 86, 3242-3246.
- [16] Sánchez-Monge, R., Gómez, L., García-Olmedo, F. and Salcedo, G. (1986) *FEBS Lett.* 207, 105-109.
- [17] Sánchez-Monge, R., Barber, D., Méndez, E., García-Olmedo, F. and Salcedo, G. (1986) *Theor. Appl. Genet.* 72, 108-113.
- [18] Sánchez-Monge, R., Gómez, L., García-Olmedo, F. and Salcedo, G. (1989) *Eur. J. Biochem.* 183, 37-40.
- [19] Lowry, V.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193, 265-275.
- [20] Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) *Anal. Biochem.* 150, 76-85.
- [21] Laemmli, U.K. (1970) *Nature* 227, 681-685.
- [22] Gültekin, H. and Heermann, K.H. (1988) *Anal. Biochem.* 172, 320-329.
- [23] Gómez, L., Sánchez-Monge, R. and Salcedo, G. (1988) *Mol. Gen. Genet.* 214, 541-546.
- [24] Matsudaira, P. (1987) *J. Biol. Chem.* 262, 10035-10038.
- [25] Sutton, R., Wrigley, C.W., Baldo, B.A. (1982) *J. Immunol. Methods* 52, 183-194.
- [26] Pfeil, T., König, W., Schwalb, U., Delespesse, G., Ulmer, W.T. (1988) *J. Allergy Clin. Immunol.* 81 (res. 388), 265.
- [27] Lee, D., Edwards, R.G., Beeson, M.F., Dewdney, J.M. (1982) *Int. Arch. Allergy Appl. Immunol.* 69, 363-367.
- [28] Buonocore, V., Petrucci, T. and Silano, V. (1977) *Phytochemistry* 16, 811-820.
- [29] Barber, D., Sánchez-Monge, R., García-Olmedo, F., Salcedo, G. and Méndez, E. (1986) *Biochem. Biophys. Acta* 873, 147-151.
- [30] Almondallal, Z., Briand, J.P. and Van Regenmortel, M.H.V. (1985) *EMBO J.* 4, 1231-1235.
- [31] Van Regenmortel, M.H.V., Muller, S., Quesniaux, V.F., Altschuh, D. and Briand, J.P. (1988) *Vaccines. New Concepts and Developments* (Kholer, M. and Loverde, P.T., eds) pp. 113-122, Longman.
- [32] Menéndez-Arias, L., Moneo, I., Domínguez, J. and Rodríguez, R. (1988) *Eur. J. Biochem.* 9, 555-560.