Biochem. J. (1992) 281, 401–405 (Printed in Great Britain)

Wheat and barley allergens associated with baker's asthma

Glycosylated subunits of the α-amylase-inhibitor family have enhanced IgE-binding capacity

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A 16 kDa protein, designated CM16*, which strongly binds IgE from baker's-asthma patients has been identified as a glycosylated form of the previously reported WTAI-CM16, which is a subunit of the wheat tetrameric α-amylase inhibitor. A glycosylated form (CMb*) of BTAI-CMb, the equivalent inhibitor subunit from barley, has been also found to have significantly enhanced IgE-binding capacity. In all, 14 purified members of the α-amylase/trypsin-inhibitor family showed very different IgE-binding capacities when tested by a dot-blot assay. The glycosylated components CM16*, CMb* and the previously described non-glycosylated 14.5 kDa allergen from barley (renamed BMAI-1) were found to be the strongest allergens.

INTRODUCTION

The inhalation of cereal flours is the cause of an occupational allergy known as baker's asthma, with a high prevalence in the baking industry [1,2]. Baker's asthma is mediated by IgE antibodies, but the identification and characterization of the responsible IgE-binding proteins (allergens) that are relevant for the development of early diagnosis tests and specific therapeutic treatments is still at an early stage. Wheat-flour proteins have been described as the most prominent allergens, particularly those from the salt-soluble fraction (albumins and globulins) with molecular masses of about 12–20 kDa [3–5].

We have recently identified and characterized several 12–15 kDa salt-soluble proteins as major allergens associated with baker's asthma, in wheat and barley flours [6,7]. All these allergens belong to a single protein family, which includes inhibitors of heterologous α-amylases and of trypsin [8]. Whereas the trypsin inhibitors are monomeric proteins, three classes of α-amylase inhibitors, namely monomeric, homodimeric and heterotetrameric, have been reported [8]. Joint consideration of amino acid sequences and genetic data has allowed us to group the subunits of α-amylase inhibitors into different subfamilies, each one associated with a set of homologous loci [8,9].

A 14.5 kDa protein has been identified as a major IgE-binding component of barley flour [6]. Although this protein is active against insect α-amylase, its monomeric or homodimeric nature still remains undetermined. In the case of wheat, Walsh & Howden [10] have located a putative allergenic peptide in the *N*-terminal region of the 0.28 (synonym WMAI-1) inhibitor. This momeric component, together with other representative subunits of the different α-amylase-inhibitor subfamilies, have been identified as prominent allergens in bread and pasta wheat flours [7]. However, an uncharacterized protein with stronger IgE-binding capacity than all the inhibitor subunits tested has been detected in both types of wheat flours [7].

We report here the delineation of such an uncharacterized protein, as well as that of the equivalent allergen of barley flour. Both components seem to be glycosylated forms of previously studied tetrameric inhibitor subunits. The present paper also documents the differential reactivity of 14 purified members of the inhibitor family against IgE from patients with baker's asthma.

MATERIALS AND METHODS

Plant material

Flour from *Triticum turgidum* L., cv. Senatore Capelli (pasta wheat; genomes AABB), *Triticum asestivum* L., cv. Chinese Spring (bread wheat; genomes AABBDD) and *Hordeum vulgare* L., cv. Bomi (genomes HH) were used in this study.

Fractionation of flour α-amylase inhibitors

Crude inhibitor preparations from flours were obtained by 0.15 M-NaCl extraction and $(NH_4)_2SO_4$ precipitation as described previously [9]. These preparations were then fractionated by non-dissociating gel filtration on Sephadex G-100, using 0.1 M-ammonium acetate, pH 6.8, as elution buffer [9,11]. Fractions corresponding to the three classes of α-amylase inhibitors (monomeric, dimeric and tetrameric) were pooled, dialysed against water and freeze-dried.

Purification of inhibitor subunits

The isolation of wheat and barley inhibitor subunits from the appropriate gel-filtration fractions was performed by preparative reverse-phase h.p.l.c. The following columns and gradients were used.

T. turgidum. Subunits WTAI-CM2, WTAI-CM3B, WTAI-CM16 and CM16* were eluted on an Ultrapore 300-5 C3 column (250 mm × 10 mm) with a linear gradient of 15–35% propan-2-ol in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min (total run 180 min). Subunit WDAI-1 (synonym 0.53 inhibitor) was isolated as described previously [12].

T. aestivum. Subunits WMAI-1 (synonym 0.28 inhibitor) and WDAI-2 (synonym 0.19 inhibitor), both encoded by the D

Abbreviations used: i.e.f., isoelectric focusing; PVDF, poly(vinylidene difluoride); s.g.e., starch-gel electrophoresis; TFA, trifluoroacetic acid.

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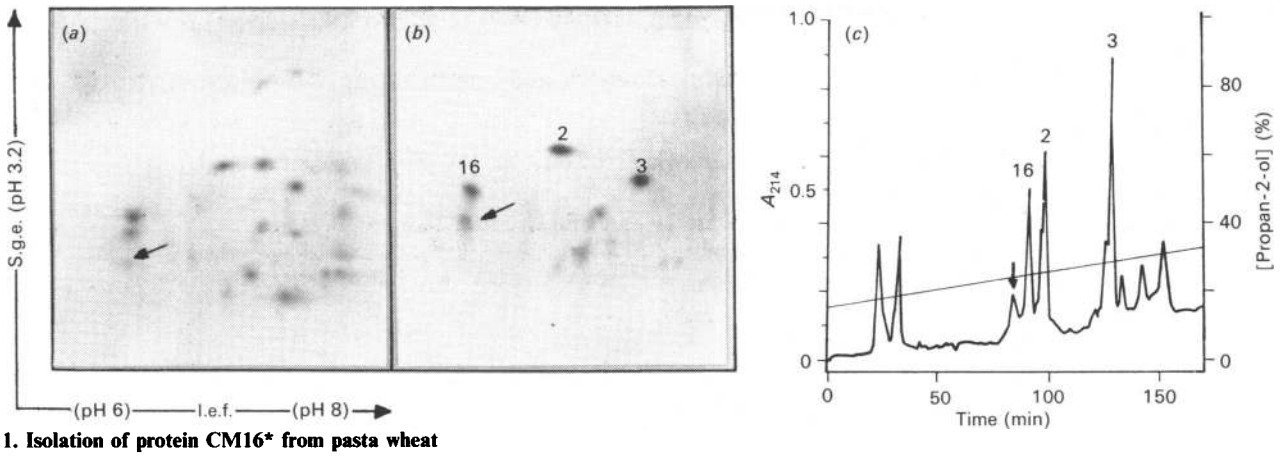

Fig. 1. Isolation of protein CM16* from pasta wheat

Two-dimensional electrophoretic maps (i.e.f. × s.g.e) of the crude inhibitor preparation (*a*) and of the 60 kDa gel-filtration fraction which includes tetrameric inhibitors (*b*) from *Triticum turgidum* L., cv. Senatore Capelli (pasta wheat). (*c*) H.p.l.c. fractionation of the tetrameric inhibitor fraction shown in (*b*). Arrows point to the position of component CM16* in both the two-dimensional maps and the h.p.l.c. elution profile. The positions of tetrameric inhibitor subunits WTAI-CM2 (2), -CM16 (16) and -CM3B (3) are also indicated.

genome, were purified from hexaploid (bread) wheat. WMAI-1 was separated on a Vydac C4 column (250 mm × 22 mm) using a two-step gradient of 20–50% (v/v) acetonitrile in 0.1% TFA (linear 20–35% gradient in 140 min; linear 35–50% gradient in 100 min) at a flow rate of 2 ml/min. WDAI-2 was purified as described by Sanchez-Monge *et al.* [13].

H. vulgare. All the barley inhibitor subunits were eluted from the corresponding gel filtration fraction on an Ultrapore 300-5 C3 columnn (250 mm × 10 mm) with a three-step gradient of 10–50% acetonitrile in 0.1% TFA (linear 10–20% gradient in 45 min; linear 20–35% gradient in 140 min; linear 35–50% gradient in 100 min) at a flow rate of 1 ml/min.

Protein cleavage and peptide separation

Proteins CM16* and CMb* were reduced and carboxymethylated as described by Craven *et al.* [14], and then subjected to enzymic hydrolysis by endoproteinase Lys-C (EC 3.4.99.30) in a 25 mM-Tris/HCl (pH 8.5)/1 mM-EDTA buffer (18 h; 37 °C; enzyme/protein ratio 1:30, w/w). The resulting peptides were fractionated by reverse-phase h.p.l.c. on a Nucleosil 300-5 C4 column (250 mm × 4.6 mm) with a linear gradient (0–70%) of acetonitrile in 0.1% TFA at a flow rate of 0.5 ml/min (the column had previously been eluted with 0.1% TFA for 5 min; total run 75 min).

Electrophoretic procedures

SDS/PAGE was carried out as described by Laemmli [15] on Bio-Rad Miniprotein II system minigels. Two-dimensional electrophoresis (i.e.f. × s.g.e.) was performed as described previously [9].

Protein determination

Protein concentration was quantified by the bicinchoninic acid ('BCA') assay described by Smith *et al.* [16].

Immunodetection

A pool of sera from nine patients with baker's asthma was used for immunodetection experiments. All sera were RAST class 3 or 4 when assayed with commercial wheat-flour discs (Phadebas-RAST kit from Pharmacia).

After SDS/PAGE the gels were soaked during 15 min in transfer buffer (50 mM-Tris/50 mM-boric acid, pH 8.3), and then electrotransferred to poly(vinylidene difluoride) (PVDF) membranes on a Bio-Rad Mini Trans-Blot cell for 60 min at 100 V. Immunodetection of IgE-binding proteins was carried out by treatment of membranes with 1:3 sera dilutions and ^{125}I-labelled anti-human IgE as described by Lughtenberg *et al.* [17].

The IgE-binding capacity of purified inhibitor subunits was also tested in dot-blot assays. Protein samples (1 μg) were solubilized in 20 mM-Tris/HCl (pH 8.3)/150 mM-NaCl buffer (TBS) containing 0.001% (w/v) SDS and 2% (v/v) β-mercaptoethanol, and then heated at 100 °C (5 min) before their adsorption to PVDF membranes equilibrated in TBS. Membrane strips were processed subsequently as above [17].

Glycoprotein assay

Glycoproteins were identified with a glycan detection kit (Boehringer), the supplier's instructions being followed. Briefly, glycoproteins (0.4 μg) were oxidized and labelled with digoxigenin before separation by SDS/PAGE and electroblotting on to nitrocellulose membranes. The incorporated digoxigenin was detected by an enzyme immunoassay using an antibody–alkaline phosphatase conjugate. Transferrin (Boehringer) and chymotrypsinogen (Sigma) were used as positive and negative controls respectively.

Amino acid sequencing

Proteins and peptides were reduced and carboxymethylated before sequencing [10]. *N*-Terminal amino acid sequences were determined by standard methods using an Applied Biosystems 477A gas-phase sequenator.

RESULTS AND DISCUSSION

Isolation of protein CM16* from wheat

The strongest IgE-binding component identified by Gomez *et al.* [7] in crude inhibitor preparations from wheat flours was an uncharacterized protein, designated 'UP'. To isolate this protein, a similar preparation from pasta-wheat flour was fractionated by gel filtration on Sephadex G-100 (results not shown). Three fractions with apparent molecular masses of about 60, 25 and 12 kDa, corresponding to tetrameric, dimeric and monomeric α-amylase inhibitors, were obtained as previously reported [9,12]. When analysed by two-dimensional electrophoresis, the UP component, named hereafter CM16*, was only detected in the gel-filtration fraction which included tetrameric inhibitors (Figs. 1*a* and 1*b*). This fraction was subjected to reverse-phase h.p.l.c. (Fig. 1*c*), and, after rechromatography of the appropriate peaks,

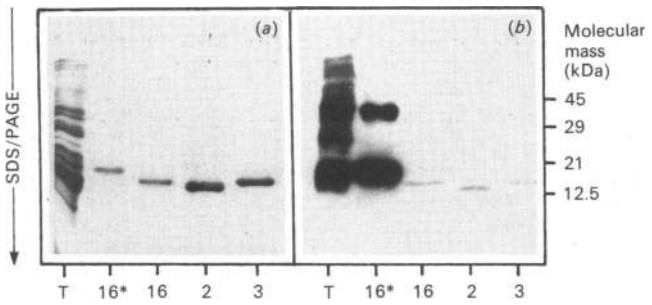

Fig. 2. IgE immunodetection of purified component CM16* and tetrameric inhibitor subunits from pasta wheat

The following samples were subjected to SDS/PAGE: crude inhibitor preparation from *T. turgidum* (T); purified protein CM16* (16*) and tetrameric inhibitor subunits WTAI-CM16 (16), -CM2 (2) and -CM3B (3). (*a*) Coomassie Blue staining. (*b*) Immunoblot of a replica of the gel in (*a*) treated with a pool of sera from baker's-asthma patients and ^{125}I-labelled anti-(human IgE) antibody.

Protein	Sequence															
	1				5				10					15		
CM16*	I	G	N	E	D	C	T	P	W	M	S	T	L	I	T	P	L	P
CMb*	V	G	S	E	D	C	T	P	W	T	A	T	P	I	T			

Fig. 3. *N*-Terminal amino acid sequences of CM16* and CMb* proteins

protein CM16* and tetrameric inhibitor subunits WTAI-CM2, -CM3B and -CM16, were obtained. Homogeneity of purified proteins was checked by two-dimensional electrophoresis (i.e.f. × s.g.e.; results not shown) and by SDS/PAGE (Fig. 2*a*).

IgE immunodetection with sera from baker's-asthma patients showed that the IgE-binding capacity of CM16* was significantly stronger than those of the three purified subunits of tetrameric inhibitors (Fig. 2*b*). A second reacting band, which was not detected by Coomassie Blue staining (Fig. 1*a*), probably corresponds to a dimer of CM16* (on the basis of its apparent molecular mass). The detection by SDS/PAGE of multimers in preparations of purified allergens has been reported in certain cases [18].

The isolated protein used for immunodetection, CM16,* was subjected to *N*-terminal amino acid sequencing (Fig. 3). No heterogeneity was found at any position. The sequence obtained was identical with that reported for WTAI-CM16 [19,20]. Joint consideration of the results presented so far indicates that both components, CM16* and WTAI-CM16, are two forms of a single protein, and have a distinctive behaviour on s.g.e., h.p.l.c. and SDS/PAGE (an apparent molecular mass around 2 kDa higher in the case of CM16*) and very different IgE-binding capacities.

Isolation of barley protein CMb*

Previous data concerning amino acid sequence similarities and gene locations of α-amylase inhibitors in wheat and barley [4,9,11] indicate the existence of three types of tetrameric inhibitor subunits. One of these types includes the equivalent components WTAI-CM16 from wheat and BTAI-CMb from barley. Taking into account the results presented above, it was predicted that a modified form of BTAI-CMb with strong IgE-binding capacity could be present in barley flour.

In order to investigate this hypothesis, the crude inhibitor preparation from barley flour was processed in a manner similar to that described for pasta wheat. After gel filtration (results not shown; see [11]) and h.p.l.c. separation of the tetrameric-inhibitor fraction, a component named CMb* and the three subunits BTAI-CMa, -CMb and -CMd were obtained (Figs. 4 and 5*b*). Component CMb* was subjected to *N*-terminal sequencing and the sequence was homogeneous (Fig. 3) and identical with that reported for BTAI-CMb [21]. IgE immunodetection with the same pool of sera used in Fig. 2(*b*) showed a great difference in IgE-binding between CMb* (the strongest reacting component) and the three purified subunits (Fig. 5*b*). As in the case of CM16*, a second band (undetected by Coomassie Blue staining) was observed in the track corresponding to CMb*.

The above results indicate that CMb* represents a modified form of BTAI-CMb from barley (in the same manner as CM16* is a modified form of its wheat equivalent WTAI-CM16).

Proteins CM16* and CMb* are glycosylated

Proteins CM16* and CMb* had higher apparent molecular masses on SDS/PAGE (Figs. 2 and 5) and lower retention times

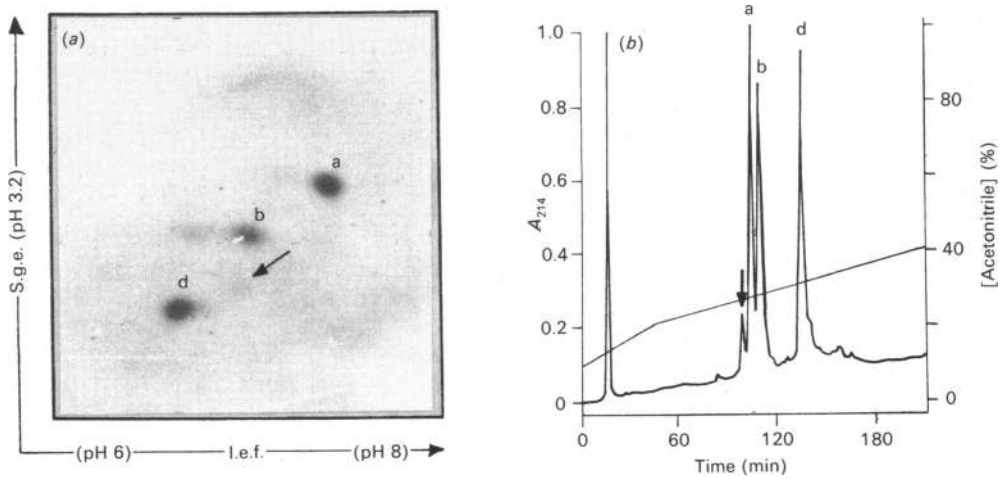

Fig. 4. Isolation of CMb* protein from barley

(*a*) Two-dimensional electrophoretic map (i.e.f. × s.g.e.) of the gel-filtration fraction corresponding to tetrameric inhibitors from *Hordeum vulgare* L., cv. Bomi. (*b*) H.p.l.c. fractionation of the tetrameric-inhibitor fraction shown in (*a*). Arrows point to the position of component CMb* in both the two-dimensional map and the h.p.l.c. elution profile. The positions of tetrameric inhibitor subunits BTAI-CMa (a), -CMb (b) and -CMd (d) are also indicated.

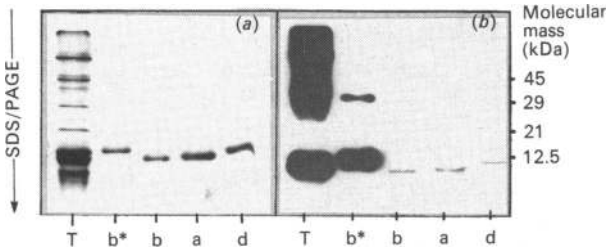

Fig. 5. IgE immunodetection of purified component CMb* and tetrameric inhibitor subunits from barley

The following samples were subjected to SDS/PAGE: crude inhibitor preparation from barley (T); purified protein CMb* (b*) and tetrameric inhibitor subunits BTAI-CMb (b), -CMa (a) and -CMd (d). (*a*) Coomassie Blue staining. (*b*) Immunoblot of a replica of the gel in (*a*) treated with a pool of sera from baker's-asthma patients and ^{125}I-labelled anti-(human IgE) antibody.

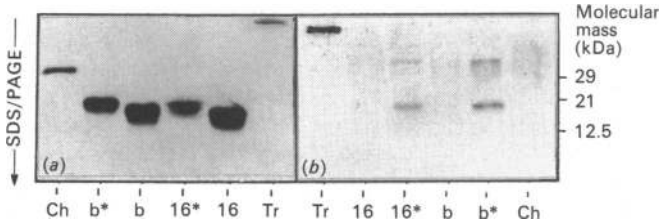

Fig. 6. Glycoprotein staining of purified components CM16* and CMb*

The following purified proteins were subjected to SDS/PAGE: CMb* (b*), BTAI-CMb (b), CM16* (16*) and WTAI-CM16 (16). Transferrin (Tr) and chymotrypsinogen (Ch) were used as positive and negative controls respectively in the glycoprotein assay. (*a*) Silver staining. (*b*) Glycoprotein staining (see the Materials and methods section).

on reverse-phase h.p.l.c. than WTAI-CM16 and BTAI-CMb respectively (Figs. 1*c* and 4*b*). Both results are compatible with a higher degree of glycosylation of the former pair. When the four purified proteins were tested in a glycoprotein assay after SDS/PAGE and electroblotting, only components CM16* and CMb* were positively stained (Fig. 6). An extra band, corresponding to the putative dimeric aggregates which were also detected in immunoblots, appeared in both cases (Figs. 2*b* and 5*b*). The glycan detection kit used has been developed to detect glycoproteins on different carriers on a qualitative basis, and its quantitative use has not yet been reported. Consequently, comparisons between Figs. 2(*b*) or 5(*b*) and 6(*b*) on a quantitative basis are not feasible. Nine other members of the inhibitor family, namely the remaining tetrameric inhibitor subunits WTAI-CM2, WTAI-CM3, BTAI-CMa and BTAI-CMd, the homodimeric inhibitors WDAI-1 (synonym 0.53) and WDAI-2 (synonym 0.19) for wheat and BDAI-1 from barley, the wheat monomeric inhibitor WMAI-1 (synonym 0.28) and the barley trypsin inhibitor BTrI-CMe, were not detected with the same glycoprotein reagent (results not shown). Previous claims that some α-amylase inhibitors (namely WMAI-1 and WDAI-2) were glycosylated [22] and that their sugar moieties play a central role in the inhibition mechanism [23] have not been confirmed using specific glycoprotein reagents. Furthermore, the production of fully active non-glycosylated WMAI-1 in *Escherichia coli* (F. García-Maroto, P. Carbonero & F. García-Olmedo, unpublished work) is in agreement with the present results. Proteins CM16* and CMb* are the only glycosylated components among the members of the inhibitor family tested so far, and are about tenfold less abundant than their non-glycosylated forms.

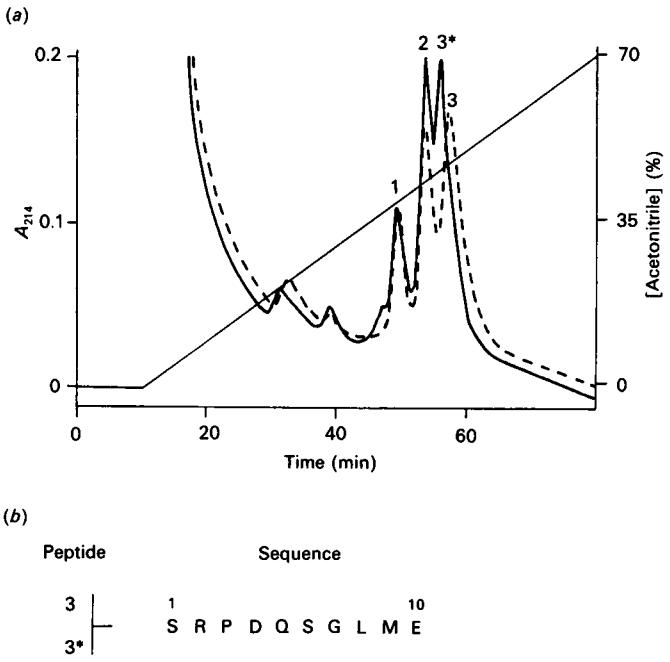

Fig. 7. Endoproteinase Lys-C peptides from components CM16* and WTAI-CM16

(*a*) H.p.l.c. fractionation of the peptides obtained after enzymic hydrolysis with endoproteinase Lys-C of components CM16* (——) and WTAI-CM16 (– – – –). Overlapping of peptides 1 and 2 from both components was checked by chromatography of the appropriate mixture. (*b*) *N*-Terminal sequence of peptides 3 and 3*. Results were identical in both cases.

The full amino acid sequence of WTAI-CM16 has been reported [20], whereas only the *N*-terminal sequence of BTAI-CMb is known [21]. Along its sequence, WTAI-CM16 has two lysine residues (nos. 41 and 67) and a single *N*-glycosylation site (NLT; residues 100–102). Further indication of the glycoprotein nature of CM16* was obtained by comparison of the peptides produced by its enzymic cleavage with endoproteinase Lys-C with those generated from WTAI-CM16 (Fig. 7). Three peptides were found in both cases, as expected from the lysine content of WTAI-CM16. Whereas peptides 1 and 2 from both components were fully coincident in h.p.l.c., peptides 3 and 3* were eluted differentially. However, these fragments showed identical *N*-terminal sequences (Fig. 7*b*). On the other hand, the sequence determined corresponded to the *N*-terminus (residues 68–77) of the predicted endo-Lys peptide that contains the single *N*-glycosylation site of WTAI-CM16. These results strongly suggest that peptides 3 and 3* differ only in a carbohydrate moeity that would be present in the fragment derived from CM16* but not in that from WTAI-CM16.

Differential IgE-binding capacity among members of the inhibitor family

The IgE-binding capacity of several purified members of the inhibitor family was tested by a dot-blot assay, and considerable differences were found (Fig. 8). The most reactive components were CM16* from wheat and CMb* and BMAI-1 from barley, followed by the dimeric barley inhibitor BDAI-1. Minor divergences with our previous results [7] are probably due to the different pools of sera and immunodetection methods used in the present work.

Protein BMAI-1 (barley monomeric α-amylase inhibitor) corresponds to the previously characterized 14.5 kDa allergen, which was found to be active against insect α-amylase [6]. This

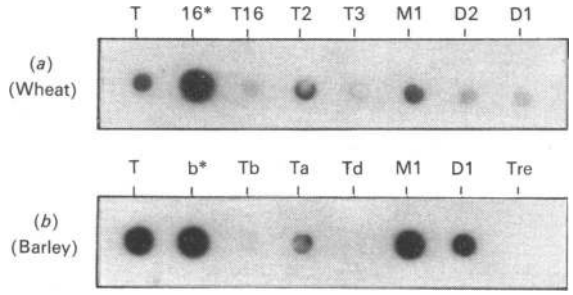

Fig. 8. IgE immunodetection by dot-blotting of purified members of the inhibitor family

Protein samples (1 μg) were adsorbed to PVDF membranes, which were subsequently treated with a pool of sera from baker's-asthma patients and ^{125}I-labelled anti-(human IgE) antibody. The following samples were tested. (*a*) Wheat: crude inhibitor preparation (T), CM16* (16*), tetrameric inhibitor subunits WTAI-CM16 (T16), -CM2 (T2) and -CM3 (T3), monomeric inhibitor WMAI-1 (M1; synonym 0.28) and homodimeric inhibitors WDAI-1 (D1; synonym 0.53) and WDAI-2 (D2; synonym 0.19). (*b*) Barley: crude inhibitor preparation (T), CMb* (b*), tetrameric inhibitor subunits BTAI-CMb (Tb), -CMa (Ta) and -CMd (Td), monomeric inhibitor BMAI-1 (M1; synonym 14.5 kDa allergen), homodimeric inhibitor BDAI-1 (D1) and trypsin inhibitor BTI-CMe (Tre).

protein, which has been so renamed because it was co-eluted with the monomeric fraction on gel filtration (results not shown), did not give a significant glycoprotein reaction under conditions in which CMb* and CM16* gave strong positive responses.

The carbohydrate moieties of CM16* and CMb* seem to be essential in order to confer a high IgE-binding capacity to these components, in comparison with the low reactivity of their corresponding deglycosylated forms. Whether or not the attached carbohydrate represents an epitope by itself remains unclear. Although glycoprotein allergens have been reported from various sources, their deglycosylation leads to very different effects: from a loss of more than 90% of the allergenic activity [24] to a slight decrease in the IgE-binding capacity [25]. However, no specific epitopes have been so far assigned to the sugar moieties.

It should be pointed out that several components of the inhibitor family which do not react as glycoproteins, such as BMAI-1 and BDAI-1, show a strong IgE-binding capacity. However, this diversity of recognized proteins might be due to the sera being polyclonal in nature and pooled from different allergic subjects.

We thank Dr. F. García-Olmedo and Dr. C. Aragoncillo for critical reading of the manuscript and Mr. J. García-Guijarro and Ms. D. Lamoneda for technical assistance. Financial support was from Comision Interministerial de Ciencia y Tecnologia (grant ALI89-0121).

Received 8 May 1991/9 July 1991; accepted 17 July 1991

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