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A major baker's asthma allergen from rye flour is considerably more active than its barley counterpart

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Abstract A rye flour protein of about 13.5 kDa, as well as its barley homologue, have been isolated. The rye component was recognized in vitro by IgE of allergic patients and provoked positive responses in 15 out of 21 baker's asthma patients (71%) when skin prick tests were performed. Its barley homologue showed no detectable in vitro reactivity and caused positive responses in only one-third of patients. Although no inhibitory activity against different α-amylases or trypsin was found for these two proteins, their N-terminal sequencing revealed considerable similarity to several members of the cereal α-amylase/trypsin inhibitor family.

Key words: Baker's asthma; Allergen; α-Amylase inhibitor family; Sequence homology; Rye

1. Introduction

Baker's asthma is a type I IgE-mediated allergic response to the inhalation of cereal flours, and represents an important occupational disease with elevated prevalence among workers of the cereal industry [1–3]. Although a number of allergens, including some additives [4], have been involved in baker's asthma, the salt-soluble proteins of flour dust seem to be the most relevant ones [5–7].

The best characterized wheat and barley flour allergens are 12–16 kDa proteins that belong to the cereal α-amylase/trypsin inhibitor family [8–10]. Most members of this family show IgE binding capacity in vitro and cause positive responses in skin prick tests. However, very different allergenic activities have been found within this family, with the glycosylated components being the most reactive allergens [9,10]. In rice, members of the α-amylase/trypsin inhibitor family are major allergens as well [11,12].

Cross-allergenicity among wheat, barley and rye flour has been reported [13,14]. In rye, two electrophoretic bands of 35 kDa and 14 kDa have been shown to include major allergens by using sera of patients sensitized to wheat [14]. However, no allergenic proteins have been purified from rye flour as yet. We report here the identification of a new member of the α-amylase/trypsin inhibitor family as a prominent rye allergen associated with baker's asthma disease.

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Abbreviations: BMAI, barley monomeric α-amylase inhibitor; BTAI, barley tetrameric α-amylase inhibitor; BTI, barley trypsin inhibitor; PVDF, poly (vinylidene difluoride); RAP, rice allergenic protein; RAST, radioallergosorbent test; SGE, starch-gel electrophoresis; TFA, trifluoroacetic acid.

2. Materials and methods

2.1. Plant material

Proteins were purified from rye (*Secale cereale* INIA C/171M) and barley (*Hordeum vulgare* cv. Bomi) flour. Disomic and ditelosomic rye–wheat (*S. cereale* cv. Imperial × *T. aestivum* cv. Chinese Spring) and barley–wheat (*H. vulgare* cv. Bomi × *T. aestivum* cv. Chinese Spring) addition lines were kindly provided by S.M. Reader (PBI, Cambridge, UK) and K.W. Shepherd (University of Adelaide, Australia), respectively.

2.2. Purification and characterization of proteins

Salt-soluble protein preparations (0.15 M NaCl extraction and $(NH_4)_2SO_4$ precipitation) enriched in inhibitors and their fractionation by gel-filtration on Sephadex G-100 under non-dissociating conditions were performed as previously described [15,16]. Fractions corresponding to dimeric inhibitors (M_r 25,000) were subjected to RP-HPLC. Sec c 1 was isolated on a Vydac-C4 column (22 × 250 mm, particle size 10 μm) eluted by a three-step linear gradient of 10–50% acetonitrile in 0.1% TFA (10–20% in 45 min, 20–35% in 140 min, 35–50% in 100 min; 2 ml/min). BDP was obtained using similar RP-HPLC conditions, except that elution was by a linear gradient of 28–48% (275 min; 1.5 ml/min). The rye α-amylase inhibitors were purified as in [16] and [17], and BMAI-1 as in [18].

Protein concentration was quantified by the bicinchoninic acid assay [19]. SDS-PAGE was performed according to Laemmli [20], and two-dimensional electrophoresis as in Gomez et al. [15]. Two-dimensional gels were stained with 0.05% (w/v) nigrosine in methanol:acetic acid:H_2O (5:1:5, v/v/v) for 16 h. N-Terminal amino acid sequences were determined by standard methods using an Applied Biosystem 477A gas-phase sequenator.

Inhibition tests of insect and human salivary α-amylases were carried out as previously reported [16,21], and anti-trypsin activity was determined according to Boisen and Djurtoft [22].

Glycoproteins were assayed after SDS-PAGE and electrotransfer to poly(vinylidene difluoride) membranes by a glycan detection kit (DIG; Boehringer) as in [9].

2.3. Chromosomal location of the gene for Sec c 1

Individual kernels of rye–wheat addition lines were delipidated with petroleum ether and then extracted with 70% (v/v) ethanol as in [17]. These extracts were fractionated by two-dimensional electrophoresis (IEF, pH 5–8 × SGE, pH 3.2).

2.4. Immunodetection

Protein samples (1 μg) in TBS buffer (20 mM Tri-HCl, 150 mM NaCl, pH 8.3) containing 0.001% (w/v) SDS and 2% (v/v) 2-mercaptoethanol were heated (100°C, 5 min) and adsorbed to PVDF membranes. Alternatively, samples were subjected to SDS-PAGE and electrotransferred to PVDF membranes as in [9]. Immunodetection of IgE-binding proteins was performed with 1:3 dilutions of a pool of sera from five baker's asthma patients (RAST class 4 to wheat and barley; Phadebas-RAST Kit, Pharmacia), and ^{125}I-labelled anti-human IgE, as reported in [23].

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2.5. In vivo diagnostic tests

The in vivo allergenic activity of Sec c 1 and BDP was investigated by skin prick tests in 21 patients (RAST ≥2 to rye) showing allergic sensitization to rye flour. All patients exhibited positive responses when prick tests and inhalation challenge tests were carried out using commercial rye flour preparations (Leti, Spain). Informed consent was obtained in all cases.

Protein samples were dissolved in 50% (v/v) glycerine, 0.9% (w/v) NaCl at a final concentration of 100 μg/ml. 5 μl of solution were applied in each case (2 repetitions per patient) using a DOME (Hollister-Stier) Bayer (1 mm T) lance (Morrow-Brown). After being transferred from the skin to adhesive paper, wheal areas were quantified by planimetry (HAFF planimeter 317). Taking into account the areas measured for atopic non-grain sensitive patients and healthy individuals, only wheals ≥7 mm^2 were considered as positive. Histamine (1%, w/v) and glycerine-NaCl were used as positive and negative controls, respectively.

3. Results

3.1. Isolation of Sec c 1 and BDP

A salt-soluble protein preparation was obtained from rye flour and fractionated by gel-filtration under non-dissociating conditions. Fractions with M_r 25,000, that include homodimeric proteins of the α-amylase/trypsin inhibitor family (see [16]), were analyzed by two-dimensional electrophoresis (Fig. 1A) and separated by RP-HPLC (Fig. 1B). One component with M_r 13,500 in SDS-PAGE (Fig. 1E), and showing a single spot on IEF × SGE (not shown), was purified using the above procedure. This protein was designated Sec c 1 according to its allergenic properties (see below) and the new WHO/IUIS Allergen Nomenclature [24].

Following similar extraction and fractionation methods, a polypeptide named BDP (barley dimeric protein) was purified from barley flour (Fig. 1C–E).

Sec c 1 and BDP are related to the cereal α-amylase/trypsin inhibitor family, but lack inhibitory activity

The N-terminal amino acid sequences of Sec c 1 and BDP are closely related (70% identity in the first 20 amino acids; Fig. 2). No heterogeneity was detected for any of the sequenced residues. Binary comparisons with members of the cereal α-amylase/trypsin inhibitor family showed sequence identities ranging from 15% to 50%. The highest values (Fig. 2) were found for trypsin inhibitors BTI and RTI [25,26], as well as rye α-amylase inhibitor-3 and its wheat (WTAI-CM2) and barley (BTAI-CMa) homologues [16]. Interestingly, major allergens from barley (BMAI [8,18]) and rice (RAP [11,12]) also showed significant sequence identity (>35%).

The above homologies prompted us to investigate the inhibitory activity of both purified proteins against α-amylases from *Tenebrio molitor* (Coleoptera), *Ephestia kuehniella* (Lepidoptera) and human saliva, as well as against bovine trypsin (enzymes that are inhibited by different members of the cereal α-amylase inhibitor family from rye and barley [16,25]). Neither Sec c 1 nor BDP showed inhibitory activity against the enzymes tested.

3.2. The genes for Sec c 1 and the rye α-amylase inhibitor-3 are located in the same chromosome arm (4RL)

The chromosomal location of the gene for Sec c 1 was determined by two-dimensional electrophoretic analysis of ethanol extracts from rye (cv. Imperial)–wheat (cv. Chinese Spring) addition lines (Fig. 3). Sec c 1 was present only in the addition lines 4R (disomic) and 4RL (ditelosomic). The same result was obtained for the rye α-amylase inhibitor-3. The positions of both components in the protein map were ascertained by co-electrophoresis of the purified proteins and the Imperial extract.

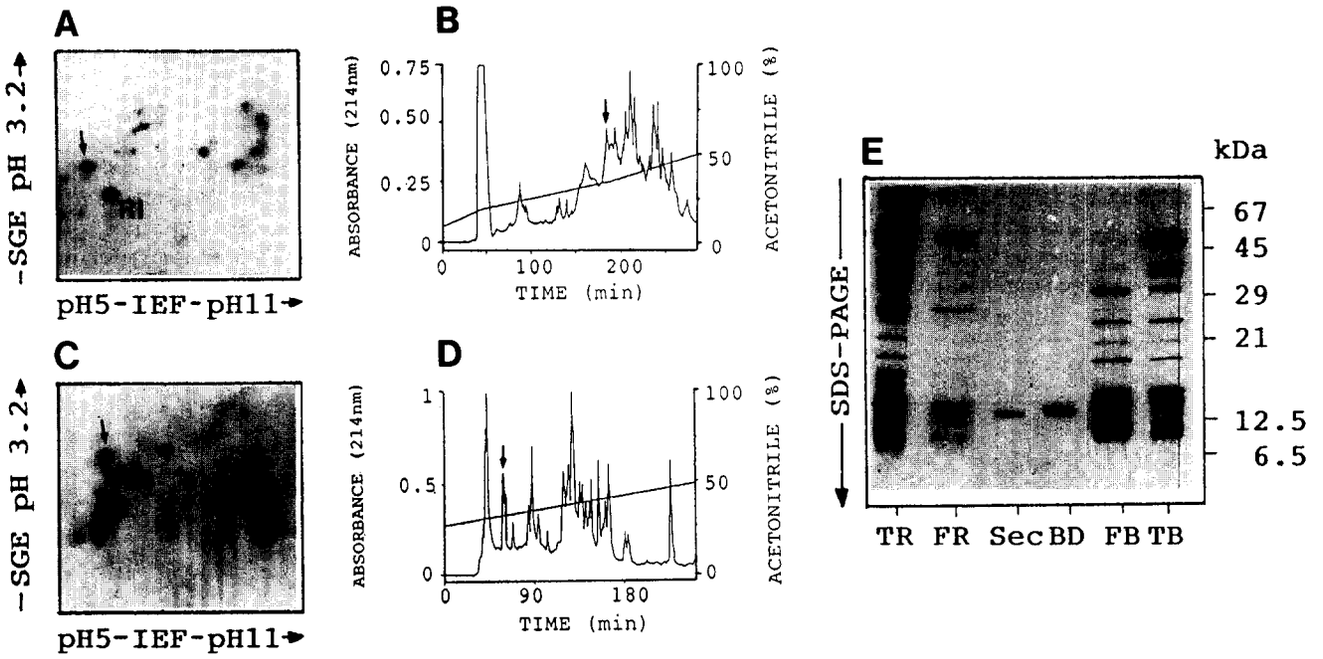

Fig. 1. Two-dimensional electrophoretic (A,C) and RP-HPLC (B,D) separations of the M_r 25,000 gel-filtration (non-dissociating conditions) fractions of salt-soluble protein preparations from rye (A,B) and barley flour (C,D). Positions of Sec c 1 (A,B), BDP (C,D) and the major rye (RI; A) and barley (BI; C) homodimeric inhibitors are indicated. Fractions containing Sec c 1 and BDP were identified after HPLC by co-electrophoresis (IEF × SGE) with the corresponding gel-filtration fractions. E) SDS-PAGE of the following samples: salt-soluble protein preparations from rye (TR) and barley (TB) flour; gel-filtration fractions with M_r ~25,000 from TR (FR) and TB (FB); purified Sec c 1 (Sec) and BDP (BD).

PROTEIN	N-TERMINAL SEQUENCE	IDENTICAL RESIDUES					
		Sec	BDP	BTI	RAI	BMAI	RAP
Sec c 1	**EQCYGESCRVGKSISNNPVPACREYV**	-	14/20	11/23	9/23	9/24	10/26
BDP	**ERDYGEYCRVGKSIPINPLP**		-	7/17	7/17	7/18	9/20
BTI	FGDSCAPGDALPHNPLRACRTYV			-	12/23	9/23	10/23
RAI-3	TGPYCYPGMGLPTKPLEGCREYV				-	11/23	10/23
BMAI	SPGEWCWPGMGYPVYPFPRCRALV					-	15/24
RAP	DHHQVYSPGEQCRPGISYPTYSLPQCRTLV						-

Fig. 2. Alignment of the N-terminal amino acid sequences of Sec c 1 and BDP with those of BTI (barley trypsin inhibitor [26]), RAI-3 (rye α-amylase inhibitor-3 [8,18]), and RAP (rice allergenic protein [12]).

Likewise, barley (cv. Betzes)–wheat (cv. Chinese Spring) addition lines were analyzed to ascertain the chromosomal location of the gene for BDP. Although BDP was present in the two-dimensional map of Betzes, it could not be detected in any of the barley–wheat addition lines, even when another electrophoretic procedure (IEF, pH 5–11 × PAGE, pH 8.8) was used.

3.3. *Sec c 1 shows IgE binding capacity in vitro and allergenic activity in vivo*

The in vitro IgE binding capacity of Sec c 1 and BDP was tested by dot-blotting and immunodetection, using a pool of sera from baker's asthma patients (RAST class 4 to wheat) and ^{125}I-labelled anti-human IgE (Fig. 4). Whereas Sec c 1 was able to bind specific IgE from allergic patients, BDP gave a negative response under the same experimental conditions. The presence of other major IgE binding components, beside Sec c 1, in the salt-soluble rye protein preparation can explain its stronger reaction with respect to Sec c 1.

To compare the responses of Sec c 1 and other salt-soluble rye proteins, including members of the α-amylase inhibitor family (inhibitors 1–3 [16]; and the rye dimeric inhibitor RDI-1 [17]), flour proteins were fractionated by SDS-PAGE, electrotransferred to PVDF membranes and probed as described above (Fig. 5). Two bands of M_r 13,000 and 32,000 were the most reactive ones. Among the main components of the 13,000 band, which include Sec c 1 and several proteins purified previously [16], only Sec c 1 showed high IgE binding capacity.

To investigate the allergenicity of Sec c 1 and BDP in vivo, skin prick tests were carried out in 21 patients allergic to cereal flour (RAST class 2–3 to rye). Sec c 1 provoked positive responses (i.e. papules with areas ≥7 mm^2) in 70% of the patients, while only one third of them reacted to BDP. Furthermore, the mean papule area (considering only positive results) was 2.7-times higher for Sec c 1 than for its barley homologue.

Since the most reactive allergens of the cereal inhibitor family are glycoproteins, we investigated the presence of oligosaccharide-side chains bound to Sec c 1 or BDP. By using a glycan detection kit and the glycosylated allergen BMAI-1 from barley [18] as a positive control, we concluded that none of the two purified proteins is glycosylated (not shown).

4. Discussion

Two salt-soluble proteins, designated Sec c 1 (allergen nomenclature) and BDP (barley dimeric protein), have been isolated from rye and barley flour, respectively. Both components co-elute in gel-filtration with the major homodimeric inhibitors of insect α-amylases previously described in these two cereal species [17,27]; Fig. 1). Furthermore, they show apparent sizes in SDS-PAGE (M_r ~13,500), isoelectric points (pI ~6), and solubility properties similar to those of dimeric inhibitors. However, neither Sec c 1 nor BDP inhibited trypsin or any of the α-amylases tested.

Sec c 1 and BDP have 70% identical N-terminal sequences, suggesting that they are equivalent components from rye and barley. Comparison of their amino acid sequences with those of different members of the cereal α-amylase/trypsin inhibitor family confirmed that Sec c 1 and BDP are homologous to this

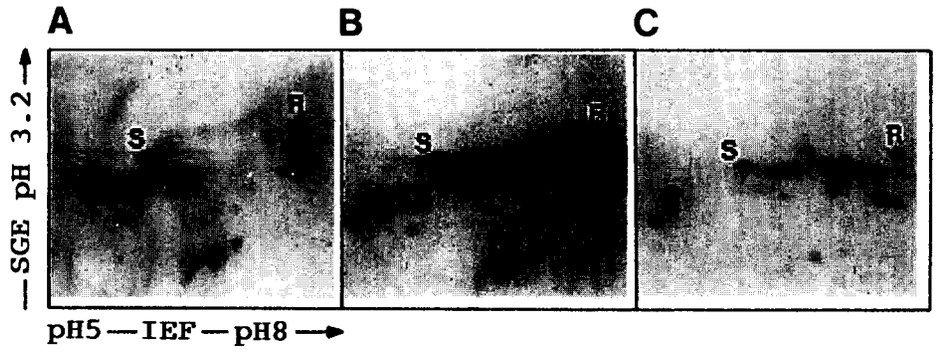

Fig. 3. Two-dimensional protein maps (IEF pH 5–8 × SGE pH 3.2) of 70% ethanol extracts from individual grains of *Secale cereale* cv. Imperial (A), a mixture of *S. cerale* cv. Imperial and *Triticum aestivum* cv. Chinese Spring (B), and Imperial-Chinese Spring ditelosomic addition line 4RL (C). Positions of Sec c 1 (S) and rye α-amylase inhibitor-3 (R) are indicated.

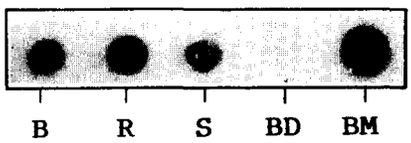

Fig. 4. IgE immunodetection by dot-blotting of the following samples: 0.15 M NaCl extract precipitated with $(NH_4)_2SO_4$ from barley (B) and rye (R) flour; purified Sec c 1 (S), BDP (BD), and the major barley allergen BMAI-1 (BM; positive control). Samples (1 μg) were adsorbed to PVDF membranes and treated with a pool of sera from baker's asthma patients and ^{125}I-labelled anti-human IgE.

protein group (Fig. 2). Major allergens from barley and rice [8,11,12,18] also show significant sequence similarity to Sec c 1 and BDP (up to 38% identical residues).

Particularly interesting is the relationship between Sec c 1 and the rye α-amylase inhibitor-3 [16]. Both proteins have 39% identical residues in their N-termini and are encoded by genes located in the same chromosome arm (4RL; Fig. 3). The two components seem to be products of a gene duplication event that has resulted in two polypeptides with different inhibitory activities. Gene duplication appears to have played a major role in the evolution of the inhibitor multigene family [25]. However, Sec c 1 and rye α-amylase inhibitor-3 represent the first case where both products do not show similar specificities towards the same hydrolytic enzyme.

Together, our data indicate that Sec c 1 and BDP must be considered as new members of the cereal α-amylase/inhibitor family, in spite of their lack of inhibitory activity against the enzymes tested. In fact, changes within the N-terminal region like those shown by Sec c 1 and BDP with regard to other active members of the inhibitor family, have been reported to produce complete inactivation or to reduce drastically the inhibitory activity when variants of a wheat monomeric inhibitor are generated by site-directed mutagenesis [28].

The homology of Sec c 1 and BDP to different cereal allergens associated with baker's asthma led us to test their IgE binding capacity using a pool of sera from patients sensitized specifically to wheat flour. In the case of salt-soluble proteins from rye, two major reactive bands with M_rs 32,000 and 13,000 were detected (Fig. 5), in agreement with previous results of Fränken et al. [14]. The main components of the 13,000 band are members of the rye α-amylase/trypsin inhibitor family [16]. Sec c 1 displays a high IgE binding capacity, conversely to the four major rye α-amylase inhibitors characterized so far [16,17]. The in vitro IgE binding capacity of BDP was negligible under our experimental conditions.

The allergenic activity of Sec c 1 has also been studied in 21 patients with high IgE to rye proteins (RAST ≥2 to rye). This purified protein provoked positive prick tests in 71% of patients, with a mean area of papule significantly higher than

Table 1
Number of reactive patients (n = 21) and mean area of papule (only positive results) produced in prick tests by purified Sec c 1 and BDP

	Positive tests[a]	Papule area (mm^2)
Sec c 1	15	41.7
BDP	7	15.3

[a]Histamine was used as positive control: 21 positive tests and 17.2 mm^2 of mean papule area.

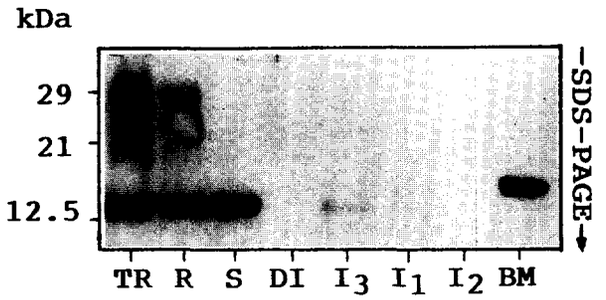

Fig. 5. IgE immunodetection after SDS-PAGE of the following samples: 0.5 M NaCl extract (TR) and 0.15 M NaCl extract precipitated with $(NH_4)_2SO_4$ (R) from rye flour; purified proteins Sec c 1 (S), rye α-amylase inhibitors-1 to -3 (I_1 to I_3), rye dimeric α-amylase inhibitor RDAI-1 (DI), and the major barley allergen BMAI-1 (BM, positive control). Immunodetection was as in Fig. 4, and 10 μg of extracts and 2 μg of purified proteins were used.

histamine (positive control). By contrast, only 33% of patients reacted with BDP.

Our results both in vitro and in vivo clearly show that Sec c 1 is a major allergen from rye flour. BDP, its barley homologue, is considerably less allergenic. Different inhibitory specificities among proteins belonging to the cereal inhibitor family have been reported previously [16].

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