

Rye γ -70 and γ -35 secalins and barley γ -3 hordein cross-react with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis

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Summary

Background Patients with wheat-dependent, exercise-induced anaphylaxis experience severe allergic reactions when exercising after ingestion of wheat. The major wheat allergen associated with these reactions is a ω -5 gliadin, and patients following a gluten-free diet have remained free of symptoms.

Objective The aim of this study was to examine whether allergens cross-reacting with wheat ω -5 gliadin are present in rye, barley and oats.

Methods Sera from 23 adult patients with wheat-dependent, exercise-induced anaphylaxis were examined. Cereal allergens cross-reacting with wheat ω -5 gliadin were identified by immunoblot inhibition. The cross-reactive allergens were purified by gel filtration and reversed-phase chromatography and submitted to amino acid sequencing. Cross-reactivity was further studied by IgE ELISA and ELISA inhibition, and *in vivo* reactivity by skin prick testing.

Results In immunoblotting rabbit anti- ω -5 gliadin antibodies bound to 70 kDa and 32 kDa proteins in rye and a 34-kDa protein in barley, but not to proteins in oats. N-terminal sequencing identified these proteins as rye γ -70 secalin, rye γ -35 secalin and barley γ -3 hordein, correspondingly. In ELISA 21/23 (91%) patients with wheat-dependent, exercise-induced anaphylaxis showed IgE antibodies to purified γ -70 secalin, 19/23 (83%) to γ -35 secalin and 21/23 (91%) to γ -3 hordein. In ELISA inhibition ω -5 gliadin inhibited over 90% of the IgE binding of pooled patient sera to solid-phase γ -secalins and γ -3 hordein. Skin prick testing gave positive reactions to γ -70 secalin in 10/15 (67%) patients, to γ -35 secalin in 3/15 (20%) patients and to γ -3 hordein in 7/15 (47%) patients.

Conclusion The results of this study show that γ -70 and γ -35 secalins in rye and γ -3 hordein in barley cross-react with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis. These findings suggest that also rye and barley may elicit symptoms in patients with wheat-dependent, exercise-induced anaphylaxis.

Keywords: anaphylaxis, exercise, allergens, gliadin, cereals, IgE, cross-reactions

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Introduction

Wheat-dependent, exercise-induced anaphylaxis is a severe form of allergy where ingestion of wheat before physical exercise induces symptoms of anaphylaxis. The severity of

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the reactions varies from local or generalized urticaria and angioedema to dyspnoea, hypotension, collapse and shock. Despite the presence of IgE antibodies to wheat, neither wheat ingestion nor exercise alone suffices to trigger the symptoms [1,2]. In addition to wheat [1–6], exercise-induced anaphylaxis has been associated with various foods, such as shellfish [7], celery [8] and nuts [3] and it is well known that exercise alone may provoke symptoms in patients with classic exercise-induced anaphylaxis [9,10].

In our previous study we identified and characterized a major allergen in wheat-dependent, exercise-induced anaphylaxis. All 18 patients showed IgE antibodies to a 65-kDa gliadin. The N-terminal sequence (32 residues) of this protein was not present in the protein sequence data banks, but it had 61% sequence identity to wheat γ -gliadin [2]. However, Kasarda et al. [11] have previously described this sequence (22 residues) as the N-terminal region of a ω -5 gliadin, and due to this we shall also refer to this allergen as ω -5 gliadin, and not γ -like gliadin, in this study.

Allergen cross-reactivity can be explained by IgE antibodies that bind to structurally similar epitopes on different proteins that are either taxonomically closely related or represent evolutionary conserved structures [12]. Because of possible cross-reactions, a gluten-free diet has been recommended for patients with wheat-dependent, exercise-induced anaphylaxis, and patients avoiding wheat, rye, barley and oats have remained free of symptoms [1,2]. However, the presence of cross-reactive allergens in these cereals has not previously been demonstrated. In this study we show that rye and barley contain allergens cross-reacting with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis.

Materials and methods

Patients

The series comprised 23 adult patients (mean age at onset 35 years, range 18–66 years) with wheat-dependent, exercise-induced anaphylaxis. Eighteen patients were from our previous study and five new patients were included [2]. All 23 patients had positive skin prick test and RAST reactions to wheat and gliadin, and IgE antibodies to ω -5 gliadin in ELISA. In addition, all 19 patients tested had positive skin prick tests to ω -5 gliadin [2]. Serum samples were obtained from 20 adult control subjects with positive skin prick test and RAST reactions to wheat: 10 had Baker's asthma or allergic occupational rhinitis from wheat flour and 10 mucocutaneous or gastrointestinal symptoms from wheat. Sera were also collected from 20 adult control subjects without wheat allergy: 10 subjects had coeliac disease and 10 recurrent urticaria. The study was approved

by the ethics committee of the Hospital for Skin and Allergic Diseases.

Production of anti- ω -5 gliadin antibodies in rabbit

Three young adult New Zealand White rabbits received three sequential injections of purified ω -5 gliadin subcutaneously into the dorsum of the metatarsal area of the rear leg at 2-week intervals. The antigen injections contained 75, 50 and 50 μ g of ω -5 gliadin in 0.2 mL 20% ethanol in phosphate-buffered saline (PBS). The first injection contained 0.2 mL of Freund's complete adjuvant and the later injections were given without the adjuvant. Serum was collected 2 weeks after the third injection and it contained high concentrations of IgG antibodies specific to ω -5 gliadin. Serum from the rabbit showing the highest titre of anti- ω -5 gliadin antibodies was chosen for recognition of structures cross-reacting with ω -5 gliadin. The immunization protocol was approved by the Institutional Animal Care and Use Committee of the National Public Health Institute.

Cereal protein extraction and purification

Rye (*Secale cereale* cv. Akusti), barley (*Hordeum vulgare* cv. Arve) and oats (*Avena sativa* cv. Veli) whole grains were ground and extracted with 0.05 mmol/L PBS with 0.15 mmol/L NaCl for 2 h at room temperature and overnight at +4° C. The samples were centrifuged (30 000 g for 20 min), and the supernatants were collected and filtered. The precipitates were extracted with 75% ethanol at room temperature overnight, centrifuged (30 000 g for 20 min), and the supernatants were collected and filtered.

The ethanol-soluble extracts containing the cereal prolamins were evaporated in a vacuum centrifuge and dissolved again in 20% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). Gel filtration was performed on a Superdex 75 HighLoad 16/60 column (Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 0.5 mL/min in 20% ACN-0.1% TFA for the ethanol-soluble extracts and in 0.05 mmol/L PBS (pH 7.0) with 0.15 mmol/L NaCl for the salt-soluble extracts. Monitoring was done at 280 nm, fractions (500 μ l) were collected and analysed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie-blue staining and immunoblotting (see below). The fractions showing positive bands in immunoblotting were further purified by reversed-phase chromatography on a Resource RPC 3 mL column (Pharmacia Biotech) using a linear gradient of ACN (20–60% in 60 min) in 0.1% TFA.

Wheat (*Triticum aestivum*, cv. Tjalve) ω -5 gliadin was purified by reversed-phase chromatography as described previously [2].

SDS-PAGE, immunoblotting and immunoblot inhibition

SDS-PAGE and immunoblotting were performed as described previously [13]. Briefly, 30 µl of cereal protein fractions from reversed-phase chromatography or gel filtration were evaporated in a vacuum centrifuge, and 15 µl of reducing buffer was added. The samples were heated at +96° C for 2 min and loaded on 12.6% acrylamide gels (approximately 2 µg/well). Electrophoresis was performed at 200 V for 45 min. The gels were then stained with Coomassie brilliant blue (Phast gel Blue R, Pharmacia Biotech) or analysed by immunoblotting, for which the proteins were transferred from the gels to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) by electroblotting for 1 h at 40 V and 1 h at 100 V. The membranes were then blocked with 5% non-fat milk powder (Valio, Helsinki, Finland) in Tris-buffered saline (TBS). Rabbit serum diluted 1 : 50 000 was added and incubated overnight at +4° C with continuous shaking. Biotinylated, species-specific whole antirabbit Ig from donkey (Amersham, Chalfont, UK) was added 1 : 5000 in 5% milk-TBS and incubated at room temperature for 30 min. Alkaline phosphatase conjugated to streptavidin (Zymed, San Francisco, CA, USA) diluted 1 : 24 000 in TBS was then added and the membranes were incubated for 30 min at room temperature. The membranes were then placed in an alkaline phosphatase colour development buffer (Bio-Rad, Richmond, CA, USA) for 10 min and rinsed with tap water.

In immunoblot inhibition the proteins were electrophoresed and transferred to PVDF membranes as described above. Rabbit serum (50 µl, diluted 1 : 5000 in TBS) was incubated with an equal volume of inhibitor solution for 1 h at room temperature. Two different concentrations of ω-5 gliadin (0.5 and 50 µg/mL) were used for inhibition. The mixture was added to the membranes in 5% non-fat milk powder in TBS, the final dilution of the rabbit serum being 1 : 50 000. Further steps were performed as described above.

IgE-ELISA and ELISA inhibition

ELISA was performed as described earlier [14]. The concentration of the purified rye (32 and 70 kDa) or barley (34 kDa) proteins was adjusted to 2 µg/mL for coating polystyrene microtiter plates (100 µl/well) (Nunc, Roskilde, Denmark). The wells were then post-coated with 1% human serum albumin in 50 mmol/L carbonate buffer, and 100 µl of patient or control serum (diluted 1 : 10) was added. Biotinylated goat antihuman IgE (Vector, Burlingame, CA, USA, diluted 1 : 1000) was added, followed by streptavidin-conjugated alkaline phosphatase (Zymed, diluted 1 : 3000) and colour substrate (Bio-Rad, Hercules,

CA, USA). The colour formed was read at 405 nm with an automated ELISA reader (Titertek Multiscan, Eflab, Espoo, Finland). The mean OD value plus three standard deviations of the control sera were used as the cut-off limit for positivity.

In ELISA inhibition, microtiter plates were coated with ω-5 gliadin or purified rye or barley proteins at a concentration of 2 µg/mL. Post-coating was performed as described above. Pooled sera (50 µl, diluted 1 : 10) from six patients (nos. 1, 5, 6, 7, 8 and 17, Table 1) with wheat-dependent, exercise-induced anaphylaxis or serum from a rabbit immunized with ω-5 gliadin (50 µl, diluted 1 : 50 000) was incubated with an equal volume of inhibitor solution. Serial 10-fold dilutions (concentrations ranging from 50 µg/mL to 0.05 ng/mL + 100 µg/mL) of ω-5 gliadin or purified rye or barley proteins were used as inhibitors. Further steps were performed as described above for ELISA.

Amino-terminal sequencing

Two rye proteins (32 and 70 kDa) and one barley protein (34 kDa) showing cross-reactivity with ω-5 gliadin in immunoblot inhibition were submitted to N-terminal amino acid sequencing performed with an ABI 494 A Procise sequencer (Applied Biosystems, Perkin Elmer, Foster City, CA, USA).

Skin prick testing

Skin prick testing with the purified rye and barley proteins (500 µg/mL in 20% ethanol-PBS) was performed in 15 patients with wheat-dependent, exercise-induced anaphylaxis. At the same time prick testing with ω-5 gliadin (50 µg/mL in 20% ethanol-PBS) was carried out. Histamine dihydrochloride (10 mg/mL, ALK, Copenhagen, Denmark) was used as a positive control and 20% ethanol-PBS as well as physiological saline as negative controls. A wheal diameter at least half the diameter of the histamine reaction was considered positive.

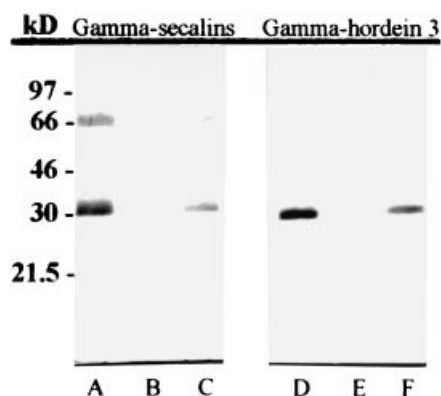
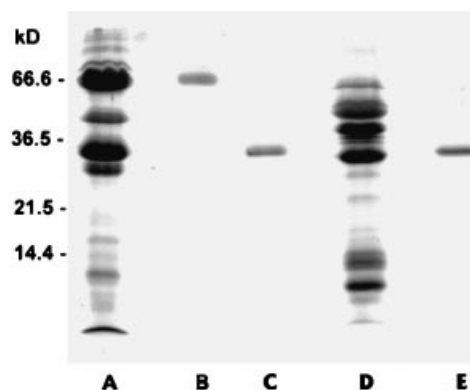
Results*Identification and characterization of rye and barley proteins cross-reacting with wheat ω-5 gliadin*

In immunoblotting the rabbit antiserum raised against ω-5 gliadin identified 32 kDa and 70 kDa proteins in the ethanol-soluble extract of rye and a 34-kDa protein in the ethanol-soluble extract of barley. The binding was completely inhibited by 50 µg/mL and partly by 0.5 µg/mL of ω-5 gliadin in immunoblot inhibition (Fig. 1). The rabbit antiserum did not bind to proteins in oats or to

Table 1. Skin prick test results in 23 patients with wheat-dependent, exercise-induced anaphylaxis

Patient No.	Sex/age at onset	Skin prick tests (wheal diameter in millimetres)				
		Histamine 10 (mg/mL)	Wheat ω -5 gliadin 50 (μ g/mL)	Rye γ -70 secalin 500 (μ g/mL)	Rye γ -35 secalin 500 (μ g/mL)	Barley γ -3 hordein 500 (μ g/mL)
1	F/30	5	7	5	4	3
2	M/22	5	7	2	0	0
3	M/28	5	12	5	0	0
4	M/31	nd	nd	nd	nd	nd
5	M/49	5	8	3	0	4
6	M/20	5	6	0	0	0
7	F/18	5	7	6	3	3
8	F/20	5	10	2	0	3
9	M/39	5	9	4	2	0
10	M/20	5	5	nd	nd	nd
11	F/36	nd	nd	nd	nd	nd
12	F/59	nd	nd	nd	nd	nd
13	M/30	6	7	nd	nd	nd
14	M/56	4	4	nd	nd	nd
15	M/30	5	5	nd	nd	nd
16	M/32	5	8	0	0	0
17	F/36	5	5	2	0	2
18	F/25	5	7	3	0	2
19	F/31	4	5	4	3	0
20	M/46	5	7	3	0	3
21	M/66	5	8	5	0	3
22	F/43	5	6	3	0	3
23	M/32	nd	nd	nd	nd	nd

nd = not done

**Fig. 1.** Immunoblot inhibition of IgG binding of rabbit anti- ω -5 gliadin antibodies to rye 70 kDa and 32 kDa proteins (lanes A-C) and barley 34 kDa protein (lanes D-F) with ω -5 gliadin. Lanes A and D, no inhibitor; lanes B and E, ω -5 gliadin 50 μ g/mL; lanes C and F, ω -5 gliadin 0.5 μ g/mL.**Fig. 2.** SDS-PAGE and Coomassie-blue staining of the purified rye (70 and 32 kDa) and barley (34 kDa) proteins. Lane A, rye total secalins; lane B, rye γ -70 secalin; lane C, rye γ -35 secalin; lane D, barley total hordeins; lane E, barley γ -3 hordein.

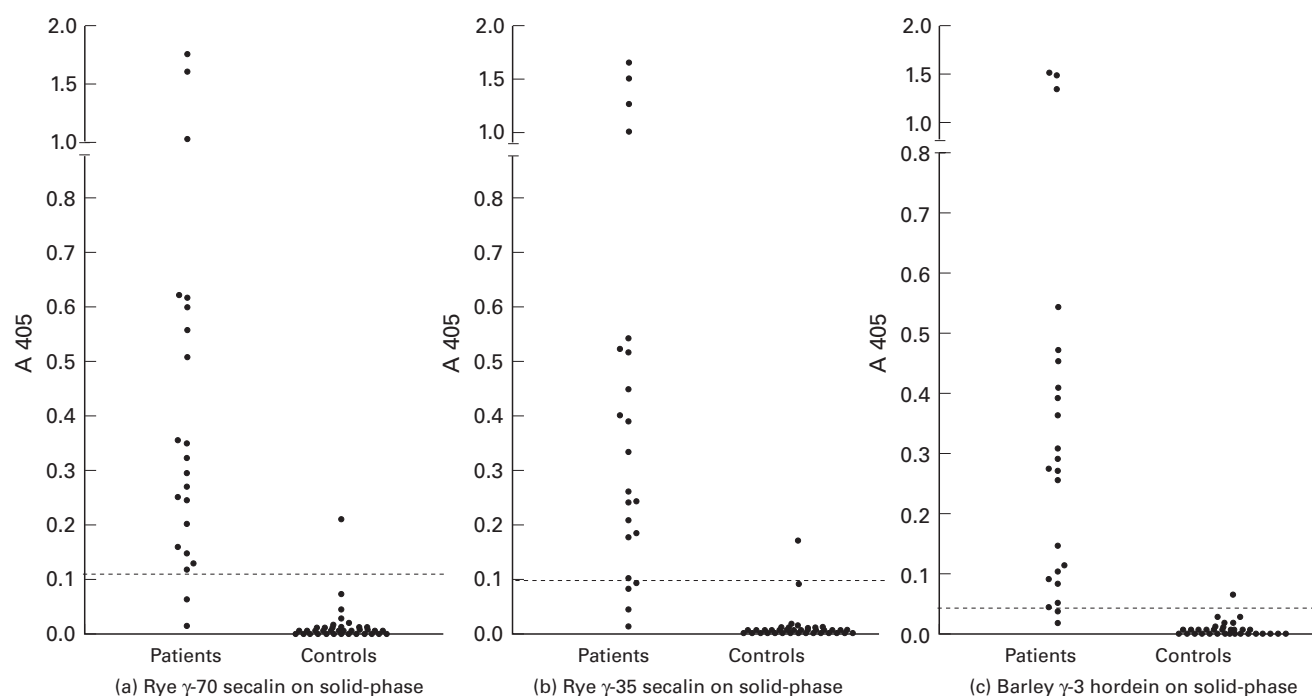


Fig. 3. IgE antibody levels to γ -70 secalin (a), γ -35 secalin (b) and γ -3 hordein (c) in ELISA (absorbancy units) in 23 patients with wheat-dependent, exercise-induced anaphylaxis and 40 controls.

proteins in the salt-soluble cereal extracts. The 32, 34 and 70 kDa proteins recognized by anti- ω -5 gliadin antibodies were also the major allergens detected by IgE from pooled patient sera.

The rye and barley proteins were purified into apparent homogeneity by gel filtration and reversed-phase chromatography (Fig. 2). Amino-terminal sequencing of the rye 70 kDa protein gave a sequence of NMQVNPSGQVQWPQQQPFPPQ (20 residues) showing 100% identity to the amino-terminus of rye γ -70 secalin. The rye 32 kDa protein gave a sequence of NMQVGPSGQVEWPQQQLPQPQQ (23 residues) showing 96% identity to rye γ -35 secalin. The amino-terminal sequence of the barley 34 kDa protein was ITTTTTFNPSGLELERPQQLFPQW (24 residues), identical to that of barley γ -3 hordein.

IgE antibodies to rye γ -70 and γ -35 secalins and barley γ -3 hordein in ELISA

In ELISA 21/23 (91%) patients with wheat-dependent, exercise-induced anaphylaxis showed IgE antibodies to γ -70 secalin, 19/23 (83%) to γ -35 secalin and 21/23 (91%) to γ -3 hordein (Fig. 3). One control patient with Baker's asthma had IgE to the rye γ -secalins and one wheat allergic patient to barley γ -3 hordein. All other 40 control subjects were negative (Fig. 3).

IgE ELISA inhibition

In ELISA inhibition ω -5 gliadin (100 μ g/mL) inhibited over 90% of the IgE binding of pooled patient sera and of the IgG binding of rabbit antiserum to solid-phase γ -70 and γ -35 secalins and γ -3 hordein. When the γ -secalins and γ -3 hordein were used as inhibitors (100 μ g/mL) and ω -5 gliadin was on solid-phase, the inhibition was about 50% in pooled patient sera (Fig. 4). γ -70 secalin inhibited over 50% of the IgG binding of rabbit antiserum to solid-phase ω -5 gliadin, but no significant inhibition was achieved with γ -35 secalin or γ -3 hordein.

Skin prick testing with rye γ -70 and γ -35 secalins and barley γ -3 hordein

Skin prick testing (500 μ g/mL) gave positive reactions to γ -70 secalin in 10/15 (67%) patients, to γ -35 secalin in 3/15 (20%) patients and to γ -3 hordein in 7/15 (47%) (Table 1). All 15 patients tested gave positive reactions to ω -5 gliadin (50 μ g/mL).

Discussion

This study shows that IgE antibodies in patients with wheat-dependent, exercise-induced anaphylaxis bind to rye and barley prolamins in addition to wheat gliadin. In ELISA 91% of the 23 patients showed IgE antibodies to

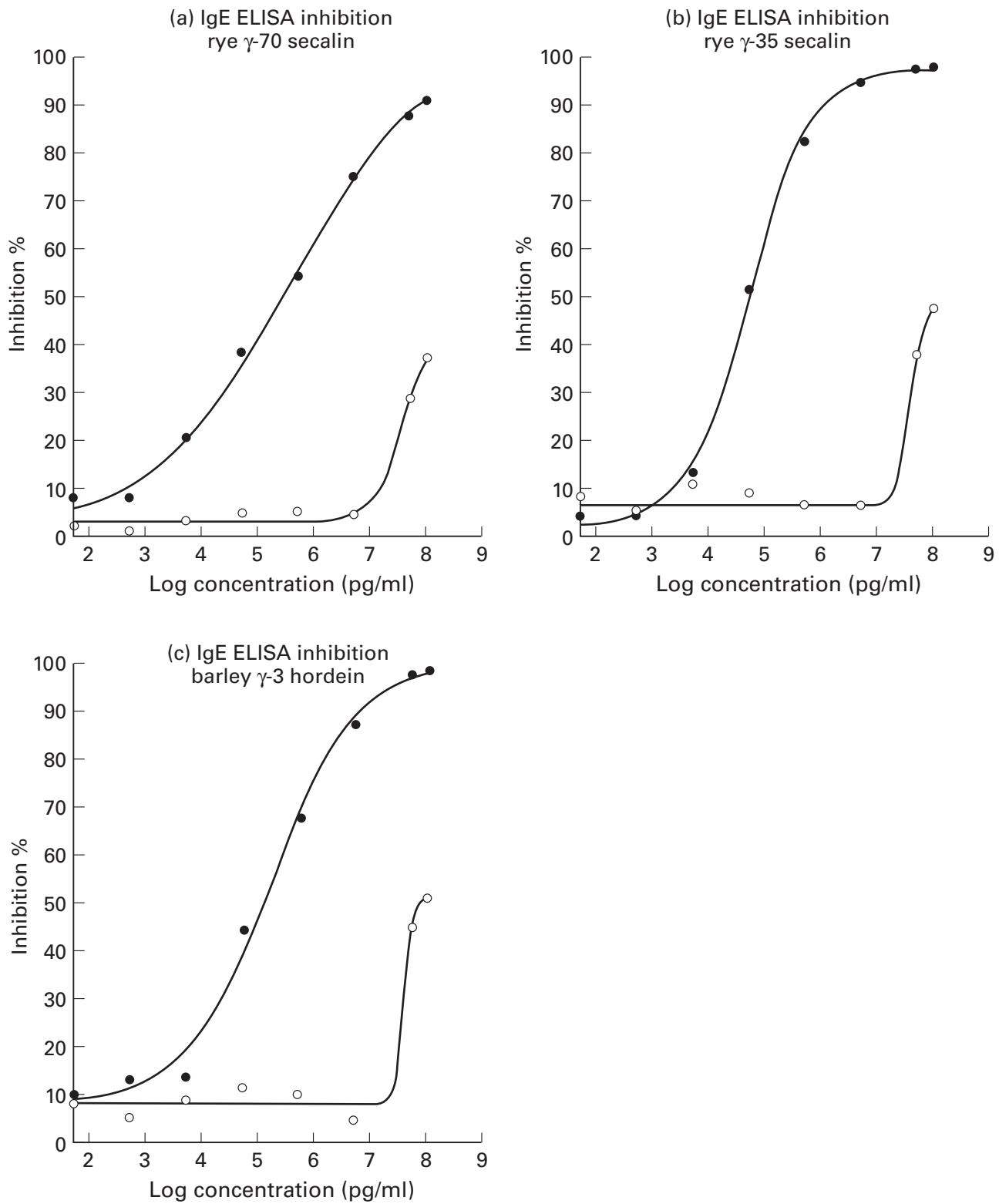


Fig. 4. Inhibition of IgE binding from pooled patient sera to solid-phase γ -70 secalin (a), γ -35 secalin (b) and γ -3 hordein (c) with ω -5 gliadin (closed circles) and vice versa (open circles).

purified γ -70 secalin and γ -3 hordein and 83% to γ -35 secalin. Skin prick testing performed in 15 patients was positive with γ -70 secalin in 10 patients, with γ -3 hordein in seven patients and with γ -35 secalin in three patients. In ELISA inhibition studies ω -5 gliadin inhibited, in a dose-dependent manner, over 90% of the binding of IgE antibodies from pooled patient sera to solid-phase γ -70 secalin, γ -35 secalin and γ -3 hordein. These results clearly demonstrate that rye γ -70 and γ -35 secalins and barley γ -3 hordein cross-react with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis.

The significance of wheat ω -5 gliadin as a major allergen in wheat-dependent, exercise-induced anaphylaxis is evident from the results of our previous study [2]. The present study suggests that rye and barley contain proteins that may share structural homology and IgE binding epitopes with wheat ω -5 gliadin. In ELISA inhibition studies IgE antibodies in the sera from patients with wheat-dependent, exercise-induced anaphylaxis showed greater affinity to ω -5 gliadin than to γ -secalins or γ -3 hordein. This supports the view that wheat ω -5 gliadin is the primary sensitizer among the proteins studied. Accordingly ω -5 gliadin gave positive skin prick test reactions at a lower concentration than γ -70 and γ -35 secalins and γ -3 hordein. The highest frequency of positive skin prick test reactions was seen for γ -70 secalin, suggesting this protein is the most important allergen cross-reacting with ω -5 gliadin. Unfortunately, no complete sequence comparisons between ω -5 gliadin and γ -secalins or γ -3 hordein can be performed, since only short amino-terminal sequences (up to 32 amino acids) are known for these proteins [2,15,16].

The ethanol-soluble cereal prolamins, i.e. gliadins in wheat, secalins in rye, hordeins in barley and avenins in oats are the major storage proteins found in the endosperm of cereal grains. They are unusually rich in proline and glutamine and there is marked sequence homology between corresponding prolamines of wheat, rye and barley, but not oat which belongs to a different tribe in the grass family (Gramineae) [17–20]. Secalins and hordeins have not previously been identified as IgE-binding allergens, but there are a few studies suggesting that wheat gliadins may be allergens in Baker's asthma [21,22]. However, in our previous study no binding of IgE to ω -5 gliadin was observed in the 16 patients with Baker's asthma [2].

In the present study we could not disclose allergens cross-reacting with ω -5 gliadin in oats, but we cannot rule out the possibility that oats may contain IgE-binding proteins other than those cross-reacting with ω -5 gliadin. The prolamin content of oats is much lower than that of wheat, rye and barley [23] and interestingly, in contrast to gliadins, secalins and hordeins, oats avenins do not seem to be harmful for coeliac patients [15, 24–26].

Due to the potentially life-threatening reactions, avoid-

ance of rye and barley seems justified in patients with wheat-dependent, exercise-induced anaphylaxis. Two patients (Nos 3 and 7, Table 1) reported that they had experienced exercise-related allergic symptoms also after ingestion of rye. Although some of the patients (e.g. patients having negative skin prick test and ELISA reactions to secalins or hordeins) may at present be able to consume rye or barley without symptoms, the risk of further sensitization to secalins or hordeins exists. This would, in turn, considerably increase the risk of developing anaphylactic episodes in the course of time. Exercise challenge tests with rye and barley would give us more information, but such tests with wheat have produced serious reactions [27,28]. On the other hand, food and exercise challenges have often failed to reproduce symptoms in patients with food-dependent, exercise-induced anaphylaxis, and hence a negative challenge test does not rule out the possibility of exercise-induced anaphylaxis [5,6,29]. Due to these obvious risks and limitations such challenges must be carefully considered in scientific and clinical works.

Previously, we have recommended a gluten-free diet for patients with wheat-dependent, exercise-induced anaphylaxis [1,2] and the results of this study support this clinical practice. Further studies are needed to investigate the molecular mechanisms behind food-dependent, exercise-induced anaphylaxis as well as the factors that make cereal prolamins act as potent allergens. Future research will also elucidate the role of oats and also indicate whether other cereals such as rice and corn contain IgE-binding proteins capable of eliciting exercise-induced anaphylactic reactions.

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