



PERGAMON



Research Section

CM3, One of the Wheat α -Amylase Inhibitor Subunits, and Binding of IgE in Sera from Japanese with Atopic Dermatitis Related to Wheat

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Abstract—The purpose of the present study was to determine whether wheat α -amylase inhibitor subunits bind IgE sera from Japanese with atopic dermatitis (AD) related to wheat. IgE in sera from eight out of the 11 patients with RAST value of 3 or 4 against wheat-bound salt-soluble proteins of wheat and the α -amylase inhibitor proteins separated by gel filtration chromatography. The subunits of tetrameric α -amylase inhibitor, CM2, CM3 and CM16 (molecular weight approximately 14 kd), known to be major allergens for baker's asthma, were purified. Then the binding activity to IgE sera from the AD patients was examined by immunoblotting. The IgE sera bound only to CM3, not to CM2 and CM16. These results suggest that CM3 may be involved in both atopic dermatitis and baker's asthma. © 2000 Elsevier Science Ltd. All rights reserved

Keywords: atopic dermatitis; α -amylase inhibitor; food allergy; IgE-ELISA; IgE-immunoblotting technique; wheat allergen.

Abbreviations: AD = atopic dermatitis; ELISA = enzyme-linked immunosorbent assay; PBS = phosphate buffered saline; PBST = phosphate buffered saline/0.05% Tween 20; RAST = radioallergosorbent test; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

INTRODUCTION

Hypersensitivity reactions have been noted in cases of wheat flour, both by inhalation and ingestion (Baldo *et al.*, 1980; Goldstein *et al.*, 1969). “Baker's asthma”, a common disease among workers with occupational exposure to cereal flour, is an allergy caused by inhalation of cereal flour. Most prominent allergens for baker's asthma have been related to the salt-soluble fraction of wheat (Fränken and König, 1991). Gomez *et al.* (1990) identified and characterized several 12–16 kd salt-soluble proteins, members of the α -amylase/trypsin inhibitor family, as major allergens associated with baker's asthma.

IgE-mediated hypersensitivity reactions for ingested foods have been an important health problem. Several hypersensitivity reactions have been reported concerned with orally ingested wheat (Hoffman *et al.*, 1975; Sampson, 1983). It was also pointed out that IgE from atopic dermatitis (AD) patients reacts with wheat and other cereal allergens (Miyakawa *et al.*, 1988). Consumption of wheat in Japan is on the increase, and there are a number of patients suspected of AD related to ingestion of wheat products (Tsubaki *et al.*, 1992; Yamada *et al.*, 1987). Several reports suggest that there are wheat proteins reactive to IgE from AD patients not only in salt-soluble fractions but also in salt-insoluble fractions, and these proteins may be associated with ingested wheat allergy (Ikezawa *et al.*, 1994; Varjonen *et al.*, 1994; Yokota *et al.*, 1992). Tsubaki

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et al. (1995) found 33–98 kD IgE-binding wheat proteins with AD patients sera in the salt-insoluble fraction by immunoblotting. Tanabe *et al.* (1996) identified an IgE-binding epitope in enzymatic hydrolysate of gluten. In contrast, Ikezawa *et al.* (1994) reported that salt-soluble proteins with molecular masses of about 14 kD bound IgE in AD patients sera, and these might be the same proteins considered to be major allergens of baker's asthma. More recently, James *et al.* (1997) identified a subunit of the wheat dimeric α -amylase inhibitor which bound IgE from patients with hypersensitivity reactions to ingested wheat proteins. However, identification and characterization of the IgE-binding proteins responsible for allergic symptoms associated with AD concerned with wheat are still at an early stage.

In this study, we purified allergens of baker's asthma from salt-soluble wheat proteins, as described by Gomez *et al.* (1990) and reactivity with IgE from AD patients' sera was examined. The results show that CM3 (14 kD), one of the subunits of α -amylase inhibitors, binds to IgE sera from AD patients.

MATERIALS AND METHODS

Sera

Sera obtained from 11 Japanese patients with AD seen at the National Minami Fukuoka Chest Hospital (Fukuoka, Japan) were used for immunodetection studies. Sera levels of total IgE and radioallergosorbent test (RAST) classes of all the patients to wheat as well as other allergens are summarized in Table 1. The age range of the patients was 1–24 yr (mean $\pm$ SE; 9.8 ± 2.6), six were male and five female (Table 1). Total IgE was 55–21,030 IU/ml (mean $\pm$ SE; 4518 ± 1864). RAST scores against wheat were four with two sera and three for the others. Also, almost all sera reacted strongly against mite, except in patient number 11. Serum from healthy donors were used as a control. Informed consent was obtained for each subject,

and all studies were done following the ethical rules of National Minami Fukuoka Chest Hospital.

Plant material

Grains of *Triticum durum*, obtained from Nippon Flour Mills Co. Ltd (Tokyo, Japan) were ground and sifted (42 mesh) in our laboratory before use.

Preparation of salt-soluble fraction

Salt-soluble wheat proteins were obtained by 150 mM NaCl extraction and $(\text{NH}_4)_2\text{SO}_4$ precipitation, as described by Gomez *et al.* (1989). A part of the precipitation was suspended in 150 mM NaCl, dialysed against distilled water, freeze-dried and stored at -20°C until use. The remaining $(\text{NH}_4)_2\text{SO}_4$ precipitation was suspended in 100 mM ammonium acetate and fractionated by gel filtration on Sephadex G-100 (3.3 ml fractions) with 100 mM ammonium acetate used as the elution buffer. Appropriate aliquots of the eluted fractions were assayed for their inhibitory activity against α -amylase from human saliva (Sigma Chemical Co., MO, USA), according to the method of Bernfeld (1955). Gel filtration fractions were pooled, dialysed against distilled water, freeze-dried and stored at -20°C until use.

Preparation of salt-insoluble fraction

Salt-insoluble fractions were obtained by the method of Tsubaki *et al.* (1991). After extraction with 150 mM NaCl, the insoluble fraction of wheat grains was washed twice with 10 mM phosphate buffer/150 mM NaCl, pH 7.4 (PBS) and then extracted with 76 mM Tris-citrate buffer/4 M urea/20 mM 2-mercaptoethanol, pH 8.6 (20 vol/wt, 30 min). After centrifugation for 20 min at 10,000 *g*, the supernatant was dialysed against PBS, freeze-dried and stored at -20°C until use.

Purification of tetrameric α -amylase inhibitor subunits

Isolations of wheat tetrameric inhibitor subunits from the salt-soluble fractions were done by reverse-phase HPLC on a μ Bondasphere 5μ C4-

Table 1. Background information and RAST studies on atopic dermatitis patients used in this study

No.	Sex	Age (yr)	Total IgE (IU/ml)	RAST values							
				Wheat	House dust	Mite	Egg white	Milk	Soy bean	Buck-wheat	Japanese cedar
1	F	23	7960	3	n.d.	6	0	n.d.	n.d.	n.d.	5
2	M	11	5590	4	n.d.	6	2	2	4	n.d.	n.d.
3	M	18	21030	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5
4	M	2	7500	3	n.d.	6	4	3	5	4	4
5	F	12	2088	3	n.d.	5	0	0	0	n.d.	n.d.
6	F	24	2083	4	n.d.	6	3	n.d.	n.d.	4	6
7	F	7	1986	3		6	1	1	3	n.d.	4
8	M	1	737	3	n.d.	6	4	2	3	n.d.	n.d.
9	F	7	613	3	n.d.	5	2	n.d.	n.d.	n.d.	5
10	M	2	62	3		3	2	2	2	3	n.d.
11	M	1	55	3	n.d.	0	3	2	2	n.d.	n.d.

F = female; M = male; n.d. = not determined.

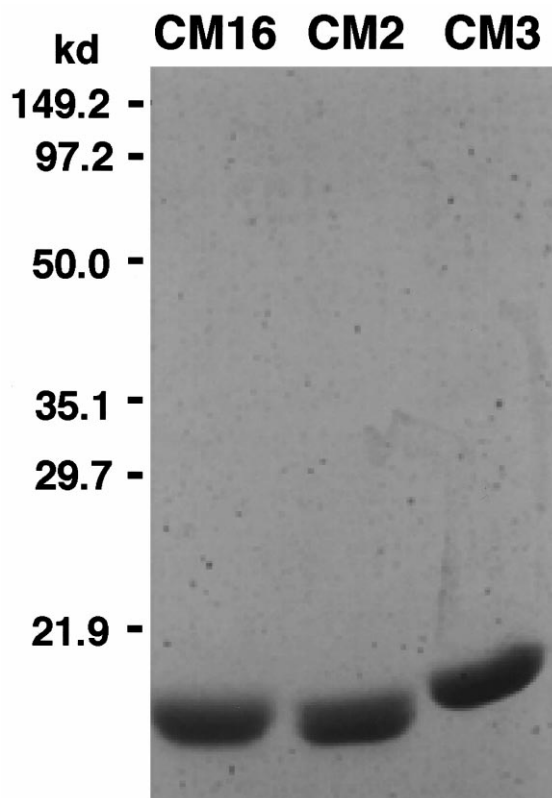


Plate 1. SDS-PAGE analysis of purified baker's asthma allergens: CM16, CM2 and CM3. Purified polypeptides (3 μ g) were assayed for immunoblot analysis.

300 Å column (150 $\times$ 3.9 mm, Nihon Waters Ltd, Tokyo, Japan), according to the method of Sanchez-Monge *et al.* (1992). Subunits were eluted with a linear gradient of 15–35% propan-2-ol in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min and each peak was subjected to N-terminal amino acid sequencing on a gas-phase protein sequence analyser (model PSQ-1; Shimadzu, Kyoto, Japan). Phenylthiohydantoin amino acids were separated by reverse-phase HPLC. The sequence data were compared for homologies to known proteins, using the BLAST algorithm (Altschul *et al.*, 1990).

Enzyme-linked immunosorbent assay (ELISA)

The extracted wheat proteins were fixed to the bottom of a 96-well microtitre plate. The preparation (10 mg protein) dissolved in 50 mM carbonate buffer, pH 9.6, was added to each well and incubation of the preparation was run for 1 hr at 37°C. Non-specific binding sites were blocked with 2% fish gelatin (Sigma Chemical Co., MO, USA) in PBS/0.05% Tween 20 (PBST), by incubating for overnight at 4°C. Sera from patients were diluted (1:3) with PBST/0.2% fish gelatin, then the diluted preparation was added to the wells followed by incubation for 1 hr at 4°C. After washing with PBST, biotin-conjugated sheep anti-human IgE (Kirkegaard & Perry Laboratories Inc., MD, USA) (1:1000) was added to the wells for 1 hr at 4°C. The

wells were washed with washing solution then avidin-bound peroxidase (Dako, Glostrup, Denmark) (1:5000) was added to the wells for 30 min at 4°C. After washing with washing solution, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (30 μ g)/0.003% H₂O₂ in 0.1 M citrate buffer, pH 4.0, was added to the wells. After leaving for 15 min at 37°C, 1.5% oxalic acid was added to terminate the reaction. The absorbance in each well was determined at 414 nm, using a microtitre reader.

Immunoblotting analysis

SDS-PAGE was carried out on a 12.5% gel as described by Laemmli (1970); samples were treated with 2-mercaptoethanol. Western blotting was performed using a semi-dry blotting apparatus. Non-specific binding was eliminated by incubating the nitrocellulose membrane (Shleicher & Schuell, Dassel, Germany) for 1 hr at room temperature in PBST/5% skim milk. For detection of specific IgE antibodies to wheat proteins, patients' sera were prepared at 1:1 dilution in PBST/0.5% skim milk. The secondary antibody, alkaline phosphatase-conjugated anti-human IgE (O.E.M Concepts Inc., NJ, USA) (1:5000), was incubated for 1 hr at 4°C, after which the immune-complexes were visualized by applying 100 mM Tris/100 mM NaCl/5 mM MgCl₂,

Table 2. N-Terminal amino acid sequences of HPLC peaks

HPLC peak	N-Terminal sequence																			Protein
1	I																			n.i.
2	G	N	C	T	P	W	M	S	M	T										CM16
3	A	I	G	N	E	D	C	T	P	W	M	A	S							CM2
4	T	G	—	Y	—	—	P	G	M	G	L	P	S	N	P	L	T			CM2
5	—	G	P	Y	C	Y	P	G	M	G	L	P	S	Q	P	L	E	G	C	R
	S	G	S	C	V	P	G	V	A	F	R	T	N	L	L	P	H	C	R	CM3

n.i. = not identified.

pH 9.5, containing 0.03% nitro blue tetrazolium and 0.017% 5-bromo-4-indolyl phosphate.

RESULTS

Characterization of α -amylase inhibitor subunits

Salt-soluble proteins extracted from *Triticum durum* were subjected to gel filtration as shown in Fig. 1. Four protein fractions with absorption peak at 280 nm were obtained. Inhibitory activities against α -amylase from human saliva were monitored to identify fractions containing tetrameric, dimeric and monomeric inhibitors. In agreement with documented data (Gomez *et al.*, 1989), three types of α -amylase inhibitors with apparent molecular masses of 60, 24 and 12 kd, which correspond to tetrameric, dimeric and monomeric inhibitors, respectively, were obtained: the tetrameric and dimeric fractions were active against α -amylase from human saliva (Fig. 1).

The fraction containing tetrameric inhibitor subunits was subjected for reverse-phase HPLC. Five HPLC peaks were numbered 1–5 in the order of elution (figure not shown), collected and subjected to N-terminal amino acid sequencing. The sequences from the peaks 2–5 corresponded with the tetrameric α -amylase inhibitor subunits, CM16,

CM2, CM2 and CM3, respectively (Table 2) (Barber *et al.*, 1986; Gautier *et al.*, 1990, 1991). Peak 4 might be a fractionation artefact of peak 3 or, since it had a slight difference in amino acid sequence from peak 3, a microheterogeneity might exist. The peaks 2 (CM16), 3 (CM2) and 5 (CM3) eluted from the HPLC revealed a single protein band on SDS-PAGE, as shown in Plate 1. In this study, CM16*, a glycosylated form of CM16 which has been identified and confirmed to be an important allergen in patients with baker's asthma (Sanchez-Monge *et al.*, 1992), was not detected by DIG glycan detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). It may be due to difference in the wheat species used.

Immunological studies of wheat protein with AD patients' sera

IgE-binding capacity of wheat extracts was examined by ELISA method with the sera from 11 AD patients (Fig. 2). The data for sera nos 1, 9 and 10 were not shown because their IgE-binding capacity was very weak for no. 1 and too weak to detect for nos 9 and 10. IgE in sera from eight out of 11 patients bound salt-soluble protein. Sera nos 2, 4, 5 and 6 seemed to bound strongly against salt-soluble protein. Sera nos 2, 5 and 6 also bound strongly

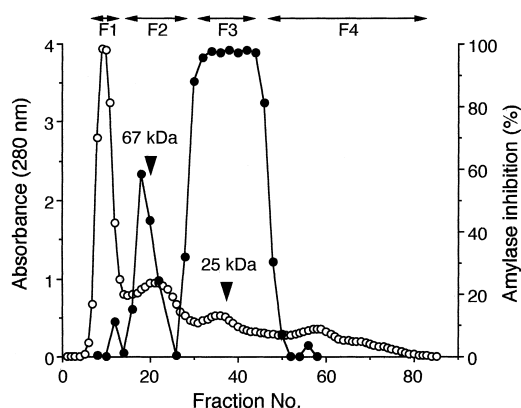


Fig. 1. The profile of the gel filtration on Sephadex G-100 of salt soluble wheat protein from *Triticum durum* (○). Bovine serum albumin (67 kd) and chymotrypsinogen (25 kd) were used to calibrate the column. The eluted fraction (protein 1 μ g) were assayed for their inhibitory activities against α -amylase from human saliva (●). Four pools were collected as indicated in the top of the figure (F1–4).

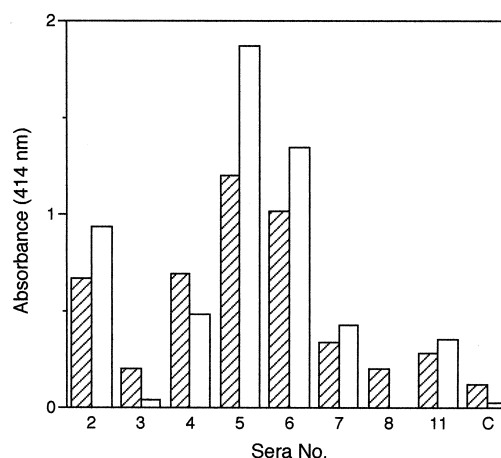


Fig. 2. IgE-ELISA values against salt-soluble fraction (▨) and salt-insoluble fraction (□) from *T. durum*. Background count was subtracted from the data. Each point is the mean of two determinations. Each fraction (protein 1 μ g) was assayed for ELISA and sera were used in fourfold dilution. C: control serum.

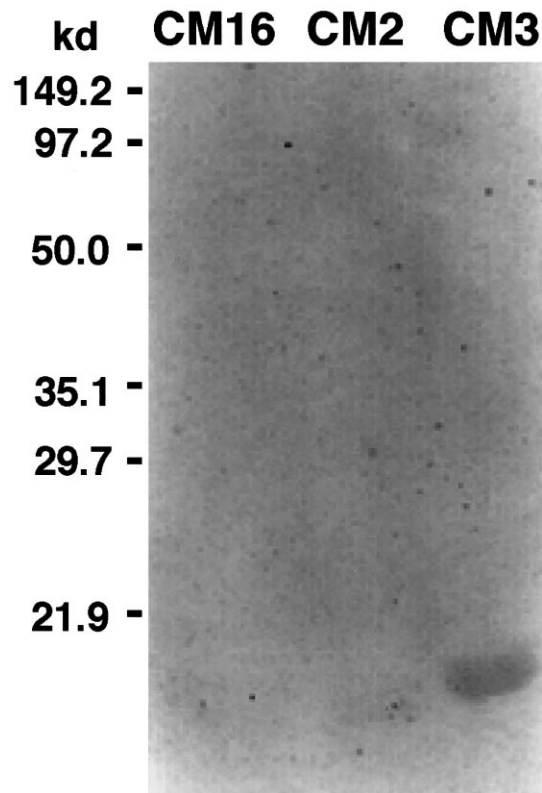


Plate 2. IgE-immunoblot analysis of purified baker's asthma allergens: CM16, CM2 and CM3, with pooled sera from AD patients in twofold dilution. Immunoblot is a replica of the gel in Plate 1. Purified polypeptides (3 μ g) were assayed for immunoblot analysis.

against salt-insoluble fraction. The sera which were positive against salt-insoluble fraction tended to react strongly with salt-soluble proteins. Furthermore, IgE-binding capacities of the proteins from the gel filtration fractions were examined by ELISA method (Fig. 3). Although the strength of the binding capacity to wheat proteins differed among the sera, all sera showed positive reactions against all the gel filtration fractions, except sera nos 8 and 11, which were negative to fraction 4. Sera nos 2, 6 and 7 showed strong binding ability against the fractions in the order of 1 to 4. IgE from sera nos 3, 4 and 5 most strongly bound to fraction 3 out of the 4 fractions.

IgE-binding ability of the purified allergens associated with baker's asthma were tested. As there was insufficient amount of sera, we pooled the sera (except nos 1, 9 and 10) and used it for immunoblotting analysis. As shown in Plate 2, the IgE sera reacted to the purified CM3; but the reactions to the purified CM2 and CM16 seemed to be very weak, if any.

DISCUSSION

The patients participating in the present study had RAST values of 3 or 4 against wheat and no symptoms of asthma. They were diagnosed to have allergic symptoms whenever ingesting wheat-con-

taining foods. Hence, we believe that they were orally sensitized with wheat, although it cannot be ruled out that they might have been sensitized through inhalation of wheat.

Little information is available regarding relevant allergens in patients with AD concerned with wheat. In baker's asthma, the most prominent aller-

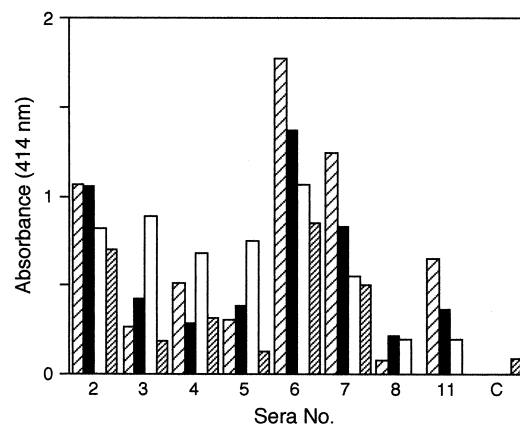


Fig. 3. IgE-ELISA values against each gel filtration fraction, F1-4 (▨, ■, □, ▤), from *T. durum*. Background count was subtracted from the data. Each point is the mean of two determinations. Each fraction (protein 1 μ g) was assayed for ELISA and sera were used in fourfold dilution. C: control serum.

gens have been found in the neutral wheat extracts. The sera of these patients mainly react with proteins with a lower molecular mass of approximately 12–20 kD (Armentia *et al.*, 1993; Gomez *et al.*, 1990; Pfeil *et al.*, 1990). In the present study, IgE levels measured by ELISA in sera of three patients were very low, or negligible, although their RAST values were three. The different results may be due to the wheat-allergen source used for each test. Whole wheat protein fraction was used for RAST, on the other hand, the salt and urea extracted fractions for ELISA. It remains a possibility that the different results were due to IgG competition on ELISA plate. However, this is unlikely because when ELISA tests were conducted via several sera dilutions, their IgE-binding activity were not detected (data not shown). Eight out of 11 AD patients sensitive to orally ingested wheat had IgE reactive to the salt-soluble fractions and gel filtration fractions enriched in α -amylase inhibitors. These results prompted us to purify the subunit of α -amylase inhibitor and to identify the components reactive to IgE sera from AD patients.

We obtained evidence that CM3 (14 kD), a component of tetrameric α -amylase inhibitor subunits, to be an allergen, as determined by using sera from patients with well defined AD. This allergen has been demonstrated to bind specific IgE in sera from adults with baker's asthma (Gomez *et al.*, 1990). In accordance to our results, James *et al.* (1997) recently identified a 15 kD allergen (0.53 inhibitor), one of the α -amylase inhibitor subunits in wheat flower extracts, using sera samples from children with defined allergic reactions after the ingestion of wheat. Taken together, these studies highlight the presence of wheat α -amylase inhibitor as a relevant allergen in patients experiencing hypersensitivity reactions after the ingestion of wheat proteins. In contrast to CM3 and 0.53 inhibitor, CM2 and CM16 did not bind patients' sera in this study. The latter two subunits are other components of tetrameric α -amylase inhibitor and have been reported to bind IgE in sera from the adult with baker's asthma (Armentia *et al.*, 1993). In contrast to inhalation, ingested allergens generally meet barriers such as blocking penetration of ingested antigens (intestinal peristalsis, intestinal mucosal coat, intestinal microvillus membrane composition), breakdown of ingested antigens (gastric acid, proteases) and immunological blocking of penetration of ingested antigens (specific IgA and reticuloendothelial system). In addition, wheat proteins are often heat denatured. These might have caused the different response of CM3 and 0.53 inhibitors from the other two subunits. To address this issue, we have prepared cDNA of CM3 for expressing the recombinant protein in bacteria, the iodinated protein was administered into rats' stomachs. We have found that the iodinated protein with molecular weight

similar to the native CM3 was recovered in the sera (unpublished data).

The present study showed that the sera from AD patients reacted with wheat proteins present in salt-insoluble fractions, indicating the existence of proteins other than α -amylase inhibitors which react to IgE sera from AD patients, Varjonen *et al.* (1994) showed that for most AD patients, there were frequent IgE-staining bands of molecular weight exceeding 26 kD for wheat proteins. Similar results were also found by Yokota *et al.* (1992) in an immunoblotting study of 15 AD patients to an urea extract of wheat.

We also found that various proteins with molecular masses of 30–40 kD in salt-soluble fraction bound to the IgE sera from AD patients (unpublished data). Therefore, low molecular weight salt-soluble proteins, 12–15 kD, can bind to IgE sera from AD patients for wheat, but it is unlikely that they are the sole IgE-binding proteins involved in AD.

In summary, the present results suggest that CM3 may be a sensitizing allergen to patients with AD concerned with wheat. However, the relationship with the clinical picture has to be further verified with subjects put on controlled diets with appropriate challenges.

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