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Degradation of Wheat Epitope Peptides for Atopic Dermatitis and Exercise-Induced Anaphylaxis by Microbial Proteases

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Abstract

Epitope peptides of wheat gliadins and glutenins for atopic dermatitis, PQQPF and QQQPP, were degraded by the microbial proteases of *Streptomyces griseus* and *Aspergillus oryzae*. The proteases of *Bacillus licheniformis* and *Bacillus amyloloiquefasiens* degraded wheat gliadins and glutenins, but had little or no effect on the epitopes. A 16-mer peptide of CSQQQQPPFSQQQPPF, which incorporated this glutenin epitope twice, was also hydrolyzed by these proteases, and no peptide containing QQQPP was shown after the reaction with *S. griseus* protease. The proteases of *S. griseus* and *A. oryzae* also degraded epitope peptides of wheat gliadins and glutenins for exercise-induced anaphylaxis, QQIPQQQ, QQFPQQQ and QQPGQ.

Discipline: Food
Additional key words: *Triticum aestivum*, allergen, enzymatic degradation

Introduction

Wheat is a source of serious food allergy worldwide, and is involved in atopic dermatitis, urticaria, and exercise-induced anaphylaxis (EIA) etc.[10,11]. Food allergy is generally caused by a cross-linking of allergens and human immunoglobulin E (IgE), and degranulation of mast cells[2,6]. The granules from mast cells contain inflammatory substances such as histamines. IgE recognizes linear 5～10 amino acid sequences of allergen proteins, which are termed epitopes. Among many wheat allergen proteins, gliadins and glutenins have been well studied[10-12]. Tanabe et al. identified a glutenin epitope (QQQPP)[15] and gliadin epitopes (PQQPF and QQPFP)[16] for atopic dermatitis. Matsuo et al. demonstrated that IgE-binding epitopes for EIA were QQIPQQQ and QQFPQQQ etc. from ω-5 gliadin[4] and QQPGQ etc. from high molecular weight (HMV) glutenin[5].

The proteases from germinated wheat seeds hydrolyzed wheat gliadins and glutenins[14], as well as the epitopes of gliadins and glutenins for atopic dermatitis[8]. Wheat flour after treatment with bacterial proteases contained partially hydrolyzed proteins and indicated a reduction in allergic responses[17], while epitopes for other wheat allergens, α-amylase inhibitors, were degraded by microbial proteases[9]. However, no degradation of the gliadin and glutenin epitopes by microbial proteases was shown. In this study, the degradation of gliadin and glutenin epitopes by microbial and food-processing proteases was analyzed by high performance liquid chromatography (HPLC) and time-of-flight mass spectrometry (TOFMS).

Materials and methods

The proteases used in this study were listed in Table 1. Although suitable peptide substrates differ among proteases, the protease activity was compared with hydrolysis of azocasein (Sigma-Aldrich, USA). The reaction mixture of 3% azocasein (0.05 mL), 0.1 M Tris-HCl buffer (pH 7.5, 0.1 mL) and enzyme solution (0.05 mL) was incubated at 37°C for 30 min, whereupon azocasein was precipitated by mixing with 20% trichloroacetic acid (0.05 mL). The soluble peptides in the supernatant after centrifugation (12,000 × g, 5 min) were measured at 366

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nm and the protein concentration was determined using a DC-protein assay (Bio-Rad, USA) and bovine serum albumin (A-2153; Sigma-Aldrich) was used as a standard protein.

A mixture of wheat gliadins and low molecular weight (LMW) glutenins, termed WGG, was prepared by a modified method of Sandiford et al.[12] Wheat flour was extracted twice with 0.5 M NaCl for 1 h, washed twice with distilled water, and then extracted with 70% ethanol. The ethanol extract was then dried by a centrifuging evaporator and dissolved with 10% dimethyl sulfoxide. WGG (2 μg) and proteases (0.2 μg) in 5 μL of 50 mM sodium citrate buffer (pH 4.5) for *A. saitoi* or Tris-HCl buffer (pH 7.5) for other proteases were incubated at 37°C for 16 h. The hydrolysis of WGG was determined by SDS-polyacryl amide gel electrophoresis (PAGE)[7] and a silver-staining kit (CosmoBio, Japan).

Custom-synthesized 5~7-mer peptides were obtained from Invitrogen (Tokyo, Japan), and a 16-mer peptide CSQQQQPPFSQQQPPF (Glu-16) was obtained from Scrum (Tokyo, Japan). Protein markers were obtained from New England Biolabs (MA, USA). The reaction mixture (50 μL) containing 0.2 or 2 μg protease, 20 μg epitope peptide, and 50 mM sodium citrate buffer (pH 4.5) for *A. saitoi* protease or Tris-HCl buffer (pH 7.5) for other proteases was incubated at 37°C for 16 h. The resting peptides were determined by HPLC on a 5C4-200 or a Navi C18-5 column (4.6 × 250 mm, Wako, Japan) with a linear gradient of acetonitrile (5% to 20 or 50% in 30 min) in 0.2% formic acid at a flow rate of 0.5 mL/min. The eluent was monitored at 214 nm. For matrix-assisted laser desorption/ionization (MALDI) TOFMS, the sample solution was mixed with a matrix solution of α-cyano-4-hydroxycinnamic acid and the positive ion mass spectrum was measured on a Voyager-DE PRO (Applied Biosystems, USA) in the reflector mode. This TOFMS was able to analyze over 500 Da of molecular mass.

Table 1. List of proteases in this study

Origin	Cat. No.	Relative activity to azocasein (%)
Streptomyces griseus	Sigma P5147	100
Bacillus licheniformis	Sigma P4860	27.2
Bacillus amyloliquefaciens	Sigma P1236	16.2
Aspergillus oryzae	Sigma P6110	14.7
Aspergillus saitoi	Sigma P2143	1.1
Bromelain from pineapple	Sigma B4782	0.5

The OD_{366} of soluble peptide was measured 3 times.

Results and discussion

1. Hydrolysis of gliadins and glutenins

The relative protease activity per protein weight to azocasein is shown in Table 1. The *S. griseus* protease indicated the highest activity, but *A. saitoi* protease and bromelain indicated very low activity. Figure 1 shows the hydrolysis of WGG by the proteases. Proteins of 30 ～45 kDa indicate gliadins and LMW glutenins (Fig. 1, lane 1). The gliadins and LMW glutenins were almost hydrolyzed by 1/10 the amount of the proteases of *B. amyloliquefaciens, B. licheniformis, A. oryzae, S. griseus* (Fig. 1, lanes 2-5), but slightly hydrolyzed by *A. saitoi* protease and bromelain (Fig. 1, lanes 2-5). The *B. amyloliquefaciens* protease-treated WGG contained partially hydrolyzed proteins, the molecular weights of which were 5-15 kDa (Fig. 1, lane 2). Such partially hydrolyzed proteins were not shown in the treatment with the proteases of *B. licheniformis, A. oryzae* and *S. griseus* (Fig. 1, lanes 3-5).

Watanabe et al. reported that wheat insoluble proteins were hydrolyzed by 1/100 the amount of actinase from *S. griseus* and collagenase from *Clostridium histolyticum* at 20°C for 24 hrs., and that the protease-treated wheat proteins indicated no allergenicity to patients' serum[17]. Since the proteases of *B. licheniformis*, *B. amyloliquefacien*s, and *A. oryzae* had the ability to hydrolyze WGG as well as the *S. griseus* protease (Fig. 1), these proteases may possibly reduce allergenicity.

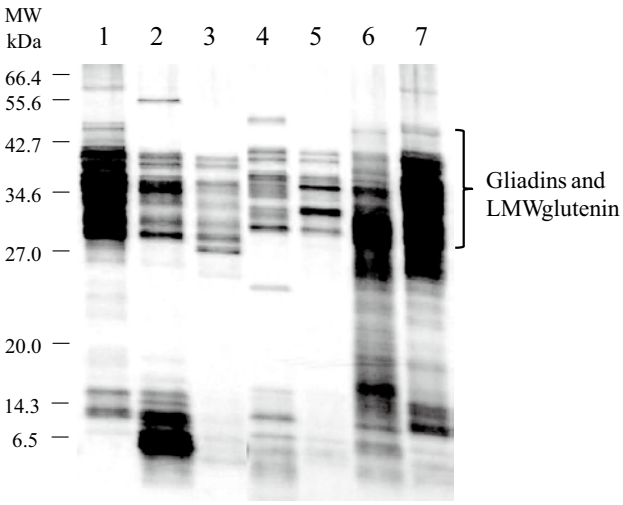

Fig. 1. Degradation of WGG by proteases
Column No. 1: no protease, 2: *B. amyloliquefasiens*, 3: *B. licheniformis*, 4: *A. oryzae*, 5: *S. griseus*, 6: *A. saitoi*, 7: bromelain.

2. Degradation of gliadin epitopes for atopic dermatitis

The enzymatic degradation of synthetic epitope peptides involved in atopic dermatitis from gliadins and glutenins is summarized in Table 2. PQQPF and QQPFP were reported as minimum IgE-binding gliadin epitopes for atopic dermatitis[16], with 5 repeats each in wheat γ-gliadin (283 amino acids)[13]. PQQPF was completely degraded by 1/50 the amount of the proteases of *S. griseus* and *A. oryzae*. QQPF was detected in the reaction mixture with 1/50 the amount of the *S. griseus* protease (Fig. 2B), but not with 1/5 the amount of the protease (Fig. 2C). The *A. oryzae* protease degraded PQQPF with a pattern resembling the *S. griseus* protease (data not shown). These results suggest that the preferable cleavage site of the epitope peptides by the proteases is between the proline and glutamine residues. The *B. licheniformis* and *B. amyloliquefacien*s proteases, for which the azocasein hydrolyzing activities were higher than the *A. oryzae* protease (Table 1), indicated less degrading activity to PQQPF. QQPFP was well degraded by 1/5 the amount of the *A. oryzae* protease, but not degraded or only to a slight extent by other proteases.

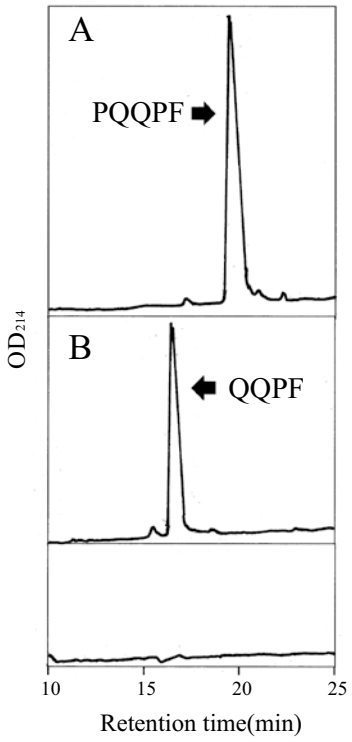

Fig. 2. HPLC of protease-treated PQQPF
A: no protease, B: 1/50 the amount of *S. griseus* protease, C: 1/5 the amount of *S. griseus* protease.

3. Degradation of LMW glutenin epitopes for atopic dermatitis

QQQPP was reported as a minimum IgE-binding epitope of glutenin for atopic dermatitis[15], and has 4 repeats in LMW glutenin (381 amino acids)[3]. QQQPP was well degraded by 1/5 the amount of the proteases from *S. griseus* and *A. oryzae*, but little degraded by the other proteases (Table 2). Due to the lower degradation of QQQPP, degradation of a longer peptide, including QQQPP, was examined. Since an allergenic 30-mer peptide, $(SQQQQPPFSQQQPPF)_2$, was isolated from the enzymatic hydrolysate of gluten[15], a synthetic 16-mer peptide Glu-16 was designed for the reaction. The N-terminal C residue of Glu-16 was added for identification of partially degraded peptides. Glu-16 was not detected after the reaction with 1/5 the amount of the proteases of *S. griseus*, *A. oryzae*, and *B. licheniformis*, and was well degraded by *B. amyloliquefaciens* protease and bromelain (Table 2).

TOFMS of the protease-treated Glu-16 samples are shown in Fig. 3. The *S. griseus* protease-treated sample indicated a main peak at *m/z* 510.5 corresponding to sodium salt of QPPF, but no peak of QQQPP (*m/z* 597.3) and its sodium salt (Fig. 3B). The *A. oryzae* protease-treated sample indicated 2 main peaks at *m/z* 1175.5 and 510.6 corresponding sodium salts of QPPFSQQQPP and QPPF (Fig. 3C). The *B. licheniformis* protease-treated sample indicated 2 main peaks at *m/z* 638.4 and 853.3 corresponding sodium salts of QQPPF and SQQQPPF (Fig. 3D). The sodium ion accompanied with the detected peptides was probably derived from NaOH, which was used for pH adjustment of the Tris-HCl buffer. QPPF and QQPPF are no longer epitopes for atopic dermatitis. These results suggest that *S. griseus* protease is the most

Table 2. Degradation of epitope peptides for atopic dermatitis

Protease	Ratio	Resting peptides (%)			
		PQQPF	QQPFP	QQQPP	Glu-16
S. griseus	1/50	0	-	87	11
	1/5	0	74	0	0
A. oryzae	1/50	0	97	59	15
	1/5	0	11	21	0
B. licheniformis	1/5	91	96	79	0
B. amyloliquefaciens	1/5	100	95	97	16
A. saitoi	1/5	92	86	95	58
Bromelain	1/5	100	100	99	9

The resting peptides were measured 3 times by HPLC.
-: Not determined.

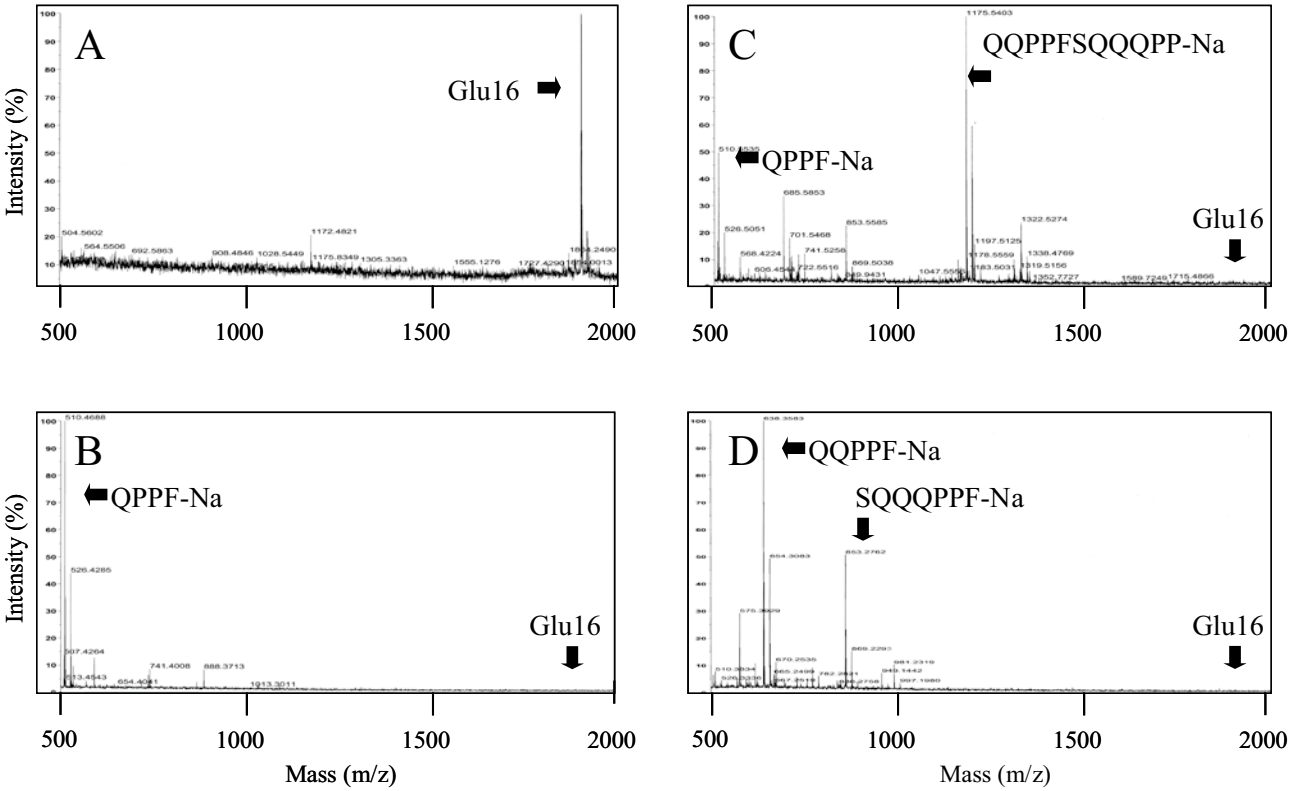

Fig. 3. Mass spectra of protease-treated Glu-16
A: no protease, B: *S. griseus*, C: *A. oryzae*, D: *B. licheniformis.*

preferable enzyme for the degradation of the LMW epitope.

The cross-linking of a peptide and multiple molecule of IgE is necessary for the degranulation of rat basophilic leukemia cells[2,6]. The partially degraded peptides of QPPFSQQQPP and SQQQPPF from Glu-16 were unable to bind to multiple molecules of IgE from wheat allergy patients, because the peptides contained only one epitope. However, it is not certain that the peptides lose the ability to elicit the degranulation of human mast cells. Therefore, LMW glutenin treated with proteases of *A. oryzae* and *B. licheniformis* possibly sustains the allergenicity.

Table 3. Degradation of epitope peptides for EIA

Protease	Ratio	Resting peptides (%)		
		QQIPQQQ	QQFPQQQ	QQPGQ
S. griseus	1/50	41	0	2
	1/5	0	-	0
A. oryzae	1/50	28	29	73
	1/5	0	0	7
B. licheniformis	1/50	92	0	-
	1/5	1	-	83
B. amyloliquefaciens	1/5	58	51	79
Bromelain	1/5	100	87	86

The resting peptides were measured 3 times by HPLC.
-: Not determined.

4. Degradation of epitopes for EIA

QQIPQQQ and QQFPQQQ were reported as minimum IgE-binding epitopes of ω-5 gliadin for EIA, each of which had 4 and 17 repeats in ω-5 gliadin (398 amino acids)[1,4]. QQFPQQQ was completely degraded by 1/50 the amount of the proteases of *S. griseus* and *B. licheniformis* and by 1/5 the amount of *A. oryzae* protease (Table 3) respectively. QQIPQQQ was almost degraded by 1/5 the amount of proteases of *S. griseus, B. licheniformis* and *A. oryzae* (Table 3). QQPGQ was reported as a minimum IgE-binding epitope of HMW glutenin for EIA, and had 35 repeats in HMW glutenin x-type subunit (819 amino acids)[5]. QQPGQ was almost degraded by the proteases of *S. griseus* and *A. oryzae*, but was slightly degraded by *B. licheniformis* protease (Table 3).

In this study, we clarified that the microbial and food-processing proteases of *S. griseus*, *A. oryzae*, and *B.*

licheniformis were effective in degrading some epitopes of wheat gliadin and glutenin for atopic dermatitis and EIA. The protease-treated wheat flour seems unsuitable for making bread and noodles requiring gluten formation from gliadins and glutenins. However, Watanabe et al. reported that the microbiological protease-treated hypoallergenic wheat flour was applicable to cupcakes and cookies[18].

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