

A Major Wheat Allergen Has a Gln-Gln-Gln-Pro-Pro Motif Identified as an IgE-Binding Epitope

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Received December 29, 1995

The minimum primary structure of the IgE-binding epitope in wheat gluten was determined as Gln-Gln-Gln-Pro-Pro. The N-terminal glutamine and the two proline residues were essential for epitopic function. The occurrence of the second glutamine residue and acetylation of the N-terminal amino group were found to exert some auxiliary functions, whereas only a lesser contribution was expected for the third glutamine residue. It was also been confirmed that acetyl-Gln-Gln-Gln-Pro-Pro bound to wheat-specific IgE antibodies in the sera of patients allergic to wheat, although it did not induce histamine release from the basophils of these patients. Taken together, we concluded that the Gln-Gln-Gln-Pro-Pro motif constituted an IgE-binding rather than immunogenic epitope and also that acetyl-Gln-Gln-Gln-Pro-Pro might act as a hapten capable of binding to specific IgE molecules. The possibility exists that this peptide will have practical application to therapy for or prevention of wheat-sensitive allergy. © 1996 Academic Press, Inc.

IgE-mediated hypersensitive responses to wheat have long been emerging an important public health problem (1, 2). α -Amylase inhibitor from a water-soluble fraction of wheat flour was identified as an one of the major allergen (3), and its IgE-binding epitope was also determined (4). However, we recently found that most patients with wheat allergy were sensitive to gluten and that the modification of gluten with collagenase and transglutaminase was effective in reducing its allergenicity (2). We have also investigated the primary structure of a major allergenic tridecapeptide isolated from an enzymatic hydrolysate of gluten and determined it to be Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Pro-Pro-Phe (5). The present study aims to clarify the minimum epitope structure of the IgE-binding site for this peptide and its biological activities.

MATERIALS AND METHODS

Reagents. Fmoc-Gln, Fmoc-Gly, Fmoc-Phe, Fmoc-Pro, Fmoc-Ser-Bu^t, and other reagents for peptide synthesis were obtained from Wako Pure Chemical Industries (Japan). The solid phase (Fmoc-NH-multipin, noncleavable type) was purchased from Cambridge Research Biochemicals (England). Ac-Gln-Gln-Gln-Pro-Pro was obtained from Peptide Institute Inc. (Japan). The acetyl peptide showed a single peak on HPLC using an ODS column and 0.1% TFA-20% CH₃CN as the eluant. Other reagents used were of reagent grade.

Peptide synthesis. Peptides were synthesized essentially according to the solid phase method (6). Briefly, Fmoc-NH-multipin was deprotected with a 1:4 mixture of piperidine and DMF at 25°C for 20 min to obtain NH₂-multipin. The coupling reaction was performed by incubating the NH₂-multipin in DMF containing Fmoc-amino acid (60 mM), HOBt (72 mM), and DIC (60 mM) at 25°C for 4 h. The same procedures were repeated alternatively for deprotection and coupling to obtain the target peptide. Deprotection of Bu^t from Ser was performed in a 19:1 mixture of TFA and anisole at 25°C for

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Abbreviations used: Ac-, acetyl; Bu^t, *t*-butyl; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; Fmoc-, 9- fluorenylmethoxycarbonyl-; HOBt, hydroxybenzotriazole; RAST, radioallergosorbent test; TFA, trifluoroacetic acid.

2.5 h. When necessary, the N-terminal amino acid was acetylated with a 50:5:1 mixture of DMF, acetic anhydride, and triethylamine at 25°C for 1.5 h. The peptide-bound multipins in a solid state were submitted to ELISA.

Hydrolysate and gluten. A 1 g portion of soft flour (Showa Sangyo Co., Japan) was treated with 10 mg of pancreatin (Wako Pure Chemical Industries) in water (10 ml) at 37°C for 6 hr. The reaction mixture was centrifuged at $5,000 \times g$ for 15 min, and the supernatant was freeze-dried to obtain a pancreatic hydrolysate of wheat flour. Gluten was prepared from the same wheat flour as previously described (2).

Patients. Peripheral blood was obtained from four patients allergic to wheat with atopic dermatitis at the National Sagami Hospital. Informed consent was obtained from all donors. The specific IgE titers of the patients were 17.7, 12.6, 9.5, and 4.3 U/ml, respectively, as estimated by RAST. Serum samples were used for ELISA after appropriate dilution. For histamine release assay, leukocyte fractions were prepared by dextran sedimentation (7).

ELISA and inhibition ELISA. ELISA was performed for each of the peptide-bound multipins by the ordinary method (8). Biotinylated anti-human IgE (Kirkegaard & Perry Lab. Inc., U.S.A.), streptavidin-peroxidase conjugate (Boehringer Mannheim, Germany), and *o*-phenylenediamine (Wako Pure Chemical Industries) were used for the assay.

Inhibition ELISA was performed by the method of Shanti *et al.* (9) as follows. Gluten (2.5 mg) was extracted with 0.1M Tris-HCl buffer (pH 8.6, 10 ml) containing 4M urea, and the extract (100 μ l) was used as the antigen in the assay. Ac-Gln-Gln-Gln-Pro-Pro was dissolved in PBS-0.05% Tween 20 at concentrations of 0.05, 0.5, 5, or 50 μ g/ml. The solution (400 μ l) was preincubated with serum (100 μ l) at 25°C for 1 h. Aliquots (100 μ l) of the Ac-Gln-Gln-Gln-Pro-Pro treated serum were used as antibody in the assay. Serum not treated with Ac-Gln-Gln-Gln-Pro-Pro was used as a control.

Histamine release assay. Histamine release was assayed according to the method of Mita *et al.* (7). Basophils (about 2×10^4 cells) from the above mentioned leukocytes were suspended in tyrode buffer (450 μ l), transferred into polypropylene tubes, and incubated at 37°C for a few minutes. Ac-Gln-Gln-Gln-Pro-Pro or the pancreatic hydrolysate of wheat flour was dissolved in tyrode buffer at concentrations of 0.01, 0.1, or 1 mg/ml, and aliquots (50 μ l) were mixed with the leukocytes. The mixtures were incubated at 37°C for 40 min and then centrifuged at $400 \times g$ for 5 min. Histamine released in each supernatant was quantified by an automated fluorometric method (10). Total histamine content of the cells was determined after treatment with 0.4N perchloric acid. The results are expressed as percentage release of total histamine corrected for spontaneous release.

RESULTS AND DISCUSSION

As reported in our previous paper (5), we isolated a major allergenic peptide from an enzymatic hydrolysate of gluten by gel filtration and reversed-phase HPLC. The primary structure of the peptide was determined to be Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe-Ser-Gln-Gln-Gln-Pro-Pro-Phe. In the present study, we first examined whether or not the motif Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe is involved in binding to specific IgE antibodies in the serum from patients allergic to wheat. The ELISA data shown in Table 1 indicate that (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₄, (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₂, and Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe bind to IgE almost equally. There was no difference between the relative ELISA values of Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe and Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe. These data suggest that the Ser-Gln-Gln-Gln-Gln-Pro-Pro-Phe motif is involved in binding with the following two possible features. One possibility is that only one IgE molecule can bind to one (Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe)₄ molecule since IgE is much larger in size than the synthetic peptide. The other possibility is that only one Ser-Gln-Gln-Gln-(Gln-)Pro-Pro-Phe motif is exposed to the aqueous phase and so able to bind to IgE.

To examine which amino acid residues in the motif are essential for binding to IgE, we attempted to replace each constituent amino acid residue in turn with Gly. When any of the asterisked amino acid residues in the sequence Ser-Gln*-Gln-Gln-Pro*-Pro*-Phe was replaced by Gly, the ELISA value dropped below the limit of detection (Table 1). These amino acid residues are therefore thought to be indispensable for binding to IgE. Table 1 also shows that Gln-Gln-Gln-Pro-Pro, which lacks the N and C terminals of Ser-Gln-Gln-Gln-Pro-Pro-Phe, gave an ELISA value equal to that obtained with the full peptide. We further examined which amino acid residues of Gln-Gln-Gln-Pro-Pro are essential for binding to IgE. The result in Table 1 indicates that the N-terminal glutamine residue and the two proline residues are essential. It is thus concluded that the IgE-binding epitope of the allergenic peptide comprises Gln-X-X-Pro-Pro, where X is a replaceable amino acid residue. The ELISA value obtained with Gln-Gly-Gln-Pro-Pro was lower by almost 30% than that obtained with Gln-Gln-Gln-Pro-Pro, and the value with non-acetylated Gln-Gln-

TABLE 1
IgE-Binding Abilities of Synthetic Peptides

Peptide	Relative ELISA value
Ac - S Q Q Q Q P P F	1.0*
S Q Q Q Q P P F	
S Q Q Q Q P P F	
S Q Q Q P P F	
Ac - S Q Q Q Q P P F	1.1
S Q Q Q P P F	
Ac - S Q Q Q Q P P F	1.1
Ac - S Q Q Q P P F	1.0
Ac - G Q Q Q P P F	1.1
Ac - S G Q Q P P F	nd**
Ac - S Q G Q P P F	0.8
Ac - S Q Q G P P F	1.0
Ac - S Q Q Q G P F	nd
Ac - S Q Q Q P G F	nd
Ac - S Q Q Q P P G	0.9
Ac - Q Q Q P P	0.9
Ac - G Q Q P P	nd
Ac - Q G Q P P	0.7
Ac - Q Q G P P	1.0
Ac - Q Q Q G P	nd
Ac - Q Q Q P G	nd
Q Q Q P P	0.6

Note. Amino acids are denoted by single-letter codes.
* Positive control.
** Not detectable.

Gln-Pro-Pro was almost half of that with acetylated Gln-Gln-Gln-Pro-Pro. From these data, the second glutamine residue of Gln-Gln-Gln-Pro-Pro and acetylation of the N-terminal amino group are both advantageous for binding to IgE.

The inhibition ELISA assay revealed that Ac-Gln-Gln-Gln-Pro-Pro binds to wheat-specific IgE in the serum of patients (Fig. 1). However, the possibility remains that other epitope structures exist, since complete inhibition was not attained. Further research is needed to define the contributions of such epitope structures to IgE binding.

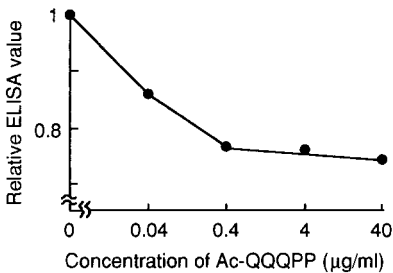


FIG. 1. Inhibition ELISA using Ac-Gln-Gln-Gln-Pro-Pro. Gluten was used as the antigen in the assay. Pooled serum of four patients allergic to wheat was preincubated at 25°C for 1 h with different concentrations (0.04, 0.4, 4, 40 μg/ml) of Ac-Gln-Gln-Gln-Pro-Pro, and the aliquots were used as antibody in the assay. For details of ELISA procedure, see *Materials and Methods*.

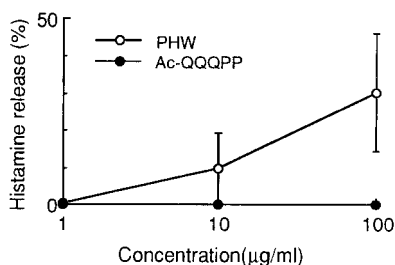


FIG. 2. Histamine release from basophils from four patients allergic to wheat. Basophils were incubated with Ac-Gln-Gln-Gln-Pro-Pro or the pancreatic hydrolysate of wheat flour (PHW) at 37°C for 40 min, and then released histamine was quantified. The results are expressed as percentage release of total histamine corrected for spontaneous release. Data are given as means $\pm$ standard errors.

Ac-Gln-Gln-Gln-Pro-Pro is so small in molecular size that the peptide itself may not be able to cross-link to IgE antibodies to cause the subsequent release of mediators such as histamine and immunoreactive leukotriene C_4 from basophils. To address this possibility, we also carried out a histamine release assay using Ac-Gln-Gln-Gln-Pro-Pro. As a positive control, a water-soluble pancreatic hydrolysate of wheat flour was used which contains the allergenic tridecapeptide (data not shown). As shown in Fig. 2, the hydrolysate induced concentration-dependent histamine release, while Ac-Gln-Gln-Gln-Pro-Pro did not lead to releasing any histamine in all cases examined. It is likely, therefore, that Ac-Gln-Gln-Gln-Pro-Pro acts as a hapten capable of binding to specific IgE molecules.

In conclusion, the minimum primary structure of the IgE-binding epitope of wheat gluten is Gln-Gln-Gln-Pro-Pro in which the N-terminal glutamine residue and the two proline residues are essential for binding. Also, the function of Ac-Gln-Gln-Gln-Pro-Pro may be of practical interest. As suggested by the report (11) that stresses the significance of using haptens as a remedy for allergies, this acetyl pentapeptide possibly can be applied as a hapten for use in the treatment or prevention of wheat-sensitive allergy.

ACKNOWLEDGMENTS

We thank Mr. Yasushi Yamazaki and Miss Yumiko Sato at Tokyo Gakugei University for their technical support.

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