Use of the phage display technique for detection of epitopes recognized by polyclonal rabbit gliadin antibodies

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Abstract A random phage heptapeptide library was screened with rabbit antibodies against wheat flour proteins comprising gliadins and a small amount of low molecular weight glutenins (gli/glu). Gli/glu antibodies isolated from the sera selected different consensus sequences (CS). All CS contained tri- to pentapeptide stretches homologous to gli/glu sequences (proposed epitopes). In α- and γ-type gliadins, these sequences are clustered in the N-terminal region recently suspected to be toxic for humans with celiac disease. Peptides with CS were synthesized and checked for reactivity. Only immune and no control rabbit sera reacted with synthetic peptides. One of eight human sera containing gliadin antibodies was reactive as well (4/8 peptides) but control sera were negative. Thus the phage display technique is useful for epitope screening of polyclonal antibodies even in the case of a group of homologous but diverse antigens.

Key words: Phage display; Peptide library; Polyclonal antibody; Gliadin; Epitope; Synthetic peptide

1. Introduction

Phage display libraries have been shown to be useful tools to investigate the interaction of ligand with defined molecules of interest. Until now, phage display libraries have mainly been used to investigate model systems applying *monoclonal* antibodies with the epitopes to be selected known in advance [1–3] or at least with antigens with known amino acid sequences [4]. The amino acid sequences selected (phagotopes) do not necessarily resemble that of the natural antigenic determinant but may mimic a discontinuous epitope (mimotopes). It would be helpful to apply the technique for elucidation of antigenic epitopes of antibodies occurring in human diseases. However, such investigations are complicated because of the *polyclonal* nature of these antibodies and because of unknown antigen. Nevertheless, recently selection of phagotopes has been described mimicking linear and structural epitopes recognized by serum-derived antibodies of rabbit [5,6] and man [7–10].

These results encouraged us to apply random peptide phage libraries to study antigenic epitopes of antibodies raised against gliadins. Gliadins are a heterogeneous group of closely related wheat storage proteins [11]. Therefore, a large number of different epitopes recognized by the antibodies may be expected. In patients with celiac disease, gliadins induce alterations of the small intestinal mucosa which may lead to malabsorption, and trigger the synthesis of antibodies against the wheat proteins and of autoantibodies. In the present report the epitope specificity was investigated of gliadin antibodies obtained by immunization of rabbits as a model for human antibodies. The aim of the study was to obtain information about the immunogenicity of the different domains of gliadins.

The results demonstrate that a number of phagotopes can be selected that are homologous to primary structures of gliadins, very probably representing antigenic epitopes, most of which are concentrated in the N-terminal region of gliadins.

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Abbreviations: CS, consensus sequence(s); gli/glu, gliadin(s)/low molecular weight (LMW) glutenin(s)

2. Materials and methods

2.1. Gliadin and gliadin antibodies

A preparation of gliadins was obtained from wheat cv. Kanzler [12]. According to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13] and immunoblot [14] the resulting product (gli/glu) contained ethanol extractable gliadins (α-, γ-, ω-type) known to be accompanied by minor amounts of gliadin-related low molecular weight (LMW) glutenins [11]. Rabbits were immunized with gli/glu [15]. Immunoglobulin G was isolated from rabbit sera by affinity chromatography on protein A. Antibodies against gli/glu (gliadin antibodies) were affinity purified using gli/glu coupled to Affigel-10 (Bio-Rad, Munich, Germany). Starting from 10 ml rabbit serum, 90 mg total IgG and 1.5 mg gliadin antibodies were obtained. Purity and specificity of isolated gliadin antibodies were checked by SDS-PAGE and immunoblot.

2.2. Phage display and sequencing

The Ph.D. Phage Display Peptide Library Kit (New England Biolabs, Schwalbach, Germany, version 1.5) was used as described [4]. After three rounds of selection, 36, 34, 67, or 65 clones (experiments 3, 9, 17, 18, respectively) were isolated and amplified. The DNA was isolated and cycle-sequenced using primers according to the instructions of the manufacturers.

2.3. Enzyme immunoassay of gliadin antibodies

Human gliadin antibodies of the IgA and IgG class were determined by enzyme immunoassay in microwells coated with gliadin as described previously [15]. The results are expressed as percentage of reactivity of a control serum provided by Labmaster (Turku, Finland). For assay of rabbit gliadin antibodies, microwells were incubated with rabbit serum (diluted 1:100 to 1:25 000) and binding of immunoglobulins was demonstrated by pig anti-rabbit immunoglobulins conjugated with peroxidase (1:2000, DAKO, code P217). Serum dilution at half maximal binding is indicated as titer.

2.4. Determination of endomysium antibodies (EmA)

IgA class EmA were determined on monkey esophagus sections (Virimmun, Frankfurt, Germany) [16].

2.5. Synthesis of cellulose-bound peptides and antibody binding studies

Peptides covalently bound to a cellulose membrane via their C-

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termini were prepared by automated spot synthesis [17] as previously described in detail [18]. After washing in methanol and buffer (TBS-T: NaCl 137 mM, KCl 2.7 mM, Tris 50.4 mM, Tween 0.05%, pH 8.0) membranes were blocked, washed in TBS-T and incubated in rabbit serum (1:100). After further washing membranes were incubated with anti-rabbit immunoglobulins conjugated with peroxidase (DAKO, code P217, 1:500 in TBS-T). After washing luminescence was measured using the Supersignal CL-HPR kit according to the instructions of the manufacturer (Pierce, Rockford, IL, USA, code 34080) on Hyperfilm-ECL (Amersham, Braunschweig, Germany). Binding was assessed qualitatively as positive (+) or negative (−).

2.6. Homology search

The database of the National Center of Biotechnology Information (NCBI Bethesda, MD, USA, release March 18, 1998) was searched using the Advanced BLAST program [19]. The data base comprised 294 713 individual sequences with a total of 88 550 842 letters and containing 43 entries for α-type gliadins with a total of 11 895 amino acids, 18 entries for γ-type gliadins with a total of 4418 amino acids, and seven entries for low molecular weight glutenins with 1914 amino acids. Only entries comprising peptides of at least 200 amino acids in length but no short fragments were considered. These gliadin/LMW glutenin (gli/glu) sequences were used for further homology search with consensus sequences (CS) obtained by phage display. Sequences were considered CS if they contained identical amino acids in at least three positions and they occurred in at least three independent phage clones. If there were several overlapping motifs identical to gli/glu sequences within the CS, priority was given to the longest sequence. Similarity of amino acids was considered according to the BLOSUM62 substitution matrix [20].

3. Results

In ELISA, immune sera of all rabbits showed a high titer of gliadin antibodies. In immunoblot, the antibodies reacted with a large number of gliadin fractions in the molecular weight range between 30 and 80 kDa and sera of the different animals produced very similar staining patterns. Serum of control rabbits showed low reactivity in ELISA and produced no staining in immunoblots (data not shown).

The amino acid composition of sequenced peptide inserts after panning with gliadin antibodies from four rabbits was different from that of the naive library. Glutamine was overrepresented by a factor of 2.7–7.7 in peptide inserts obtained from three rabbits, which is in accordance with the high proportion of this amino acid in gliadins. For all four rabbits the proline content was between 14 and 21%, which is close to that reported for gliadins.

The sequences of the selected peptides were grouped into CS (Table 1). Antibodies from different rabbits selected up to three different CS. All CS extended over the complete phagotope of seven amino acids. In two of the CS (9/1 and 18/1), tetrapeptide motifs were found in different positions within the heptapeptide. Between 4 and 18% of all sequences could not be grouped into a CS. After panning on immunoglobulins derived from serum of a control rabbit no CS was obtained.

CS were compared with gli/glu sequences. Several tri-, tetra-, and pentapeptide stretches of CS were found that were homologous to gli/glu sequences. From CS 18/1 two possible epitopes were postulated dependent on substitution of Q in the epitope by E in the phagotope.

The frequency with which the proposed epitopes occurred in a gli/glu molecule was calculated as the mean number per gli/glu molecules. Further, the total data base was screened for homology with peptide motifs showing homology with gliadins. A ratio was calculated between total data base entries and those entries comprising the group of prolamins. The ratio was small for tripeptide motifs (<0.007) but high for tetra- and pentapeptide motifs (mean value 0.2 or 0.5, respec-

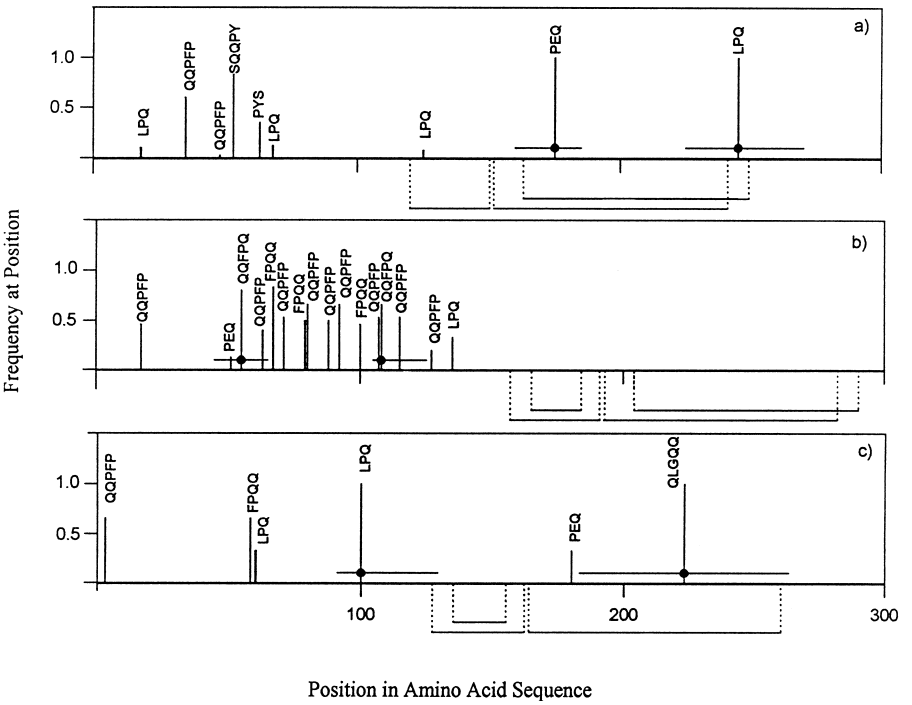

Fig. 1. Distribution of postulated epitopes in α-type and γ-type gliadin, and LMW glutenin sequences. The occurrence of different epitopes at specified positions in the sequence is indicated as mean frequency by bars (horizontal lines indicate variation of epitope position within the different α-type and γ-type gliadin and LMW glutenin species). A mean frequency of 1 means that all currently known sequences of α-type and γ-type gliadins and LMW glutenins contain the proposed epitope once at the specified position. Dotted lines below the *x*-axis show the location of disulfide bridges. In α- and γ-type gliadins epitopes are clustered in the N-terminal region.

tively). 84% of all entries containing the sequence QQPFP were prolamins.

Peptides with CS were synthesized and tested for binding to rabbit sera (Table 2). None of the immune sera recognized all of the peptides. Sera which were used for selection of phage bound to at least one of the selected peptides. One peptide was recognized by all sera applied for biopanning experiments, two peptides were recognized by none. Testing of further sera positive for gliadin antibodies revealed binding of two sera to two peptides. Rabbit control sera reacted with none of the peptides.

To investigate if sequences recognized by rabbit antibodies

Table 1
Consensus sequences (CS) selected in biopanning experiments and postulated epitopes in gli/glu

	Experiment	*n*	Amino acid sequence								Mean frequency[a]		
											α	γ	LMW
3	CS	30	G	**P**	**Y**	**S**	L	L	L				
	I		97	**100**	**100**	**93**	60	100	70				
	S		97	**100**	**100**	**95**	63	100	97				
	Epitope			**P**	**Y**	**S**					0.33	0.00	0.00
	*I**			**100**	**100**	**93**							
	*S**			**100**	**100**	**95**							
9	CS 1	10	W	M	**E**	**Q**	**P**	**F**	**P**				
	I		20	30	**90**	**50**	**100**	**100**	**100**				
	S		20	60	**100**	**80**	**100**	**100**	**100**				
	Epitope				**Q**	**Q**	**P**	**F**	**P**		0.67	4.16	0.57
	*I**				**10**	**50**	**100**	**100**	**100**				
	*S**				**100**	**80**	**100**	**100**	**100**				
	CS 2	16	L	**Q**	**Q**	**F**	**P**	**E**	L				
	I		56	**75**	**100**	**100**	**100**	**100**	69				
	S		69	**75**	**100**	**100**	**100**	**100**	75				
	Epitope			**Q**	**Q**	**F**	**P**	**Q**			0.00	1.22	0.00
	*I**			**75**	**100**	**100**	**100**	**0**					
	*S**			**75**	**100**	**100**	**100**	**100**					
17	CS 1	47	N	**T**	**Q**	**Q**	**P**	**W**	Q				
	I		28	**100**	**100**	**79**	**100**	**40**	26				
	S		47	**100**	**100**	**79**	**100**	**74**	38				
	Epitope			**S**	**Q**	**Q**	**P**	**Y**			0.81	0.00	0.00
	*I**			**0**	**100**	**79**	**100**	**15**					
	*S**			**100**	**100**	**79**	**100**	**91**					
	CS 2	11	T	Q	E	W	**L**	**P**	**Q**				
	I		45	91	100	27	**100**	**100**	**100**				
	S		45	100	100	27	**100**	**100**	**100**				
	Epitope						**L**	**P**	**Q**		1.27	0.17	1.42
	*I**						**100**	**100**	**100**				
	*S**						**100**	**100**	**100**				
	CS 3	7	**Q**	**I**	**G**	**Q**	**Q**	V	P				
	I		**100**	**71**	**100**	**100**	**57**	43	47				
	S		**100**	**100**	**100**	**100**	**86**	43	57				
	Epitope		**Q**	**L**	**G**	**Q**	**Q**				0.00	0.00	1.00
	*I**		**100**	**29**	**100**	**100**	**57**						
	*S**		**100**	**100**	**100**	**100**	**86**						
18	CS 1	34	Y	W	P	**F**	**P**	**E**	**Q**	G			
	I		9	12	21	**100**	**100**	**100**	**91**	41			
	S		21	18	21	**100**	**100**	**100**	**91**	41			
	Epitope						**P**	**E**	**Q**		0.95	0.11	0.43
	*I**						**100**	**100**	**91**				
	*S**						**100**	**100**	**91**				
	Epitope					**F**	**P**	**Q**	**Q**		0.00	2.44	0.57
	*I**					**100**	**100**	**0**	**91**				
	*S**					**100**	**100**	**100**	**91**				
	CS 2	20	A	H	**M**	**N**	**P**	S	P				
	I		32	100	**100**	**47**	**37**	21	21				
	S		37	100	**100**	**74**	**37**	58	21				
	Epitope				**M**	**N**	**P**				0.00	0.33	0.00
	*I**				**100**	**47**	**37**						
	*S**				**100**	**74**	**37**						

I: Percentage of the most common amino acid at a specified position in the CS. If there were two amino acids with the same frequency, the amino acid that had a higher number of similar amino acids at the respective position was further considered. *S*: Percentage of amino acids similar or identical to the most common amino acid (positive according to the BLOSUM62 substitution matrix). *I**: Percentage of amino acids in the consensus phagotope identical to the corresponding amino acid in the gli/glu sequences. *S**: Percentage of amino acids at a specified position of the phagotope similar to gli/glu. Peptide motifs in CS matching gli/glu sequences are printed in bold. *n*: Number of clones selected with CS.
[a]Mean frequency of epitope motifs per molecule of α-type and γ-type gliadins and of LMW glutenins. CS 1 from experiment 18 comprised eight amino acids because the consensus occurred in shifted positions within the heptapeptide.

Table 2
Reactivity of rabbit immune sera against eight different synthetic peptides with sequences according to the consensus sequences from the phage display experiments

Serum	Use in biopanning experiment no.	GPYSLLL	WMEQPFP	LQQFPEL	NTQQPWQ	TQEWLPQ	QIGQQVP	YWPFPEQG	AHMNPSP
R60a	3	+[a]	+	−	+	−	+	+	−
R46	9	−	+[a]	+[a]	−	−	−	+	−
R60b	17	+	+	−	+[a]	−[a]	−[a]	+	−
R61	18	−	+	+	−	−	−	+[a]	−[a]
R22	−	−	−	−	+	−	−	−	−
R47	−	−	−	+	+	−	−	−	−
R63	−	−	−	−	−	−	−	−	−
R69	−	−	−	+	−	−	−	−	−

Gliadin antibodies from the upper four sera were used for biopanning experiments.
[a]Sequence selected from corresponding antibodies during biopanning. Eight rabbit control sera were tested as well for reactivity with the peptides with negative results.

Table 3
Reactivity of human sera against eight different synthetic peptides corresponding to the consensus sequences from the phage display experiments

Serum	IgA gliadin antibodies	IgG gliadin antibodies	IgA EmA	GPYSLLL	WMEQPFP	LQQFPEL	NTQQPWQ	TQEWLPQ	QIGQQVP	YWPFPEQG	AHMNPSP
H356	174	49	+	−	−	−	−	−	−	−	−
H542	135	68	+	−	−	−	−	−	−	−	−
H496	79	0	+	−	−	+	−	+	+	−	+
H1267	172	47	+	−	−	−	−	−	−	−	−
H180	232	47	+	−	−	−	−	−	−	−	−
H1370	40	165	−	−	−	−	−	−	−	−	−

Five human control sera were tested as well for reactivity with the peptides with negative results.

are epitopes for human gliadin antibodies as well, the reactivity of human sera positive for gliadin antibodies was compared with human control sera (Table 3). Only one of the gliadin antibody positive sera bound to four of the peptides. Interestingly, AHMNPSP, which was not reactive with rabbit sera, was recognized by human sera, too. Human control sera did not bind.

4. Discussion

The presence of linear epitopes in gli/glu was expected because all rabbit sera recognized proteins in ethanolic flour extracts after reduction and treatment with SDS. In keeping with that, several short contiguous stretches of 3–5 amino acids were selected that were homologous with gli/glu sequences. The degree of homology between the selected phagotopes and antigenic molecules was comparable with recent observations [6,8,9].

The significance of the selected CS as gli/glu sequences can be recognized from their frequency in gli/glu molecules. Further, for the homologous tetra- and pentapeptide motifs most of the entries in the total data base were connected with gliadins and with related prolamins from related cereal species like rye, barley, and oats. Taken together this strongly implies that selected phagotopes mimic gli/glu sequences.

Selected linear regions in CS matched well homologous regions in gli/glu sequences. However, the tripeptide homology of GPYSLLL, TQEWLPQ, and AHMNPSP with gli/glu may be too small to account for binding. In these peptides, several amino acids at positions outside the region of homology to gli/glu were selected. Thus, amino acids may contribute to the epitope more distant from the linear array but brought into the close vicinity by the room structure of the antigen. Alternatively, tripeptide homology may exist purely by chance with the single amino acids as constituents of a mimotope.

However, a contribution of discontinuous epitopes to the antigenic repertoire of gliadin antibodies cannot be ruled out. To discover epitopes comprising a large number of discontinuous antibody binding points, libraries with longer peptide inserts may be better suited [21].

Several stretches of CS are homologous to more than one region in gliadin molecules. This is due to the repetitive structure of N-terminal domain Ib of gli/glu [10]. Thus, FPQQ and QQPFP are contained in γ-type gliadins on average three and four times, respectively. Therefore, the question arises if corresponding antibodies recognize only one or all of the homologous regions. Furthermore, QQPFP is found in α-type gliadins and LMW glutenins, too. Specificity for only one region would mean that amino acids in the neighborhood are important for recognition as well. Reactivity with more than one region of homology would mean a repetitive epitope structure and imply a high effectivity of binding of antibodies. Screening of binding sites on proenkephalin revealed more than one epitope region recognized by monoclonal antibody due to the repetitive structure of the protein [21].

Experiments with synthetic peptides demonstrate that the selected linear sequences represent structures recognized solely by sera obtained after immunization of rabbits with gliadin but not by control sera. This confirms the specificity of the selected peptide sequences as gli/glu epitopes. The sera already used in phage display experiments recognized a larger number of peptides in the luminescence assay than four further randomly chosen sera. This finding could be due the fact that the eight peptides were preselected by biopanning. This suggests

the existence of further antigenic epitopes recognized by gliadin antibodies in the latter sera. Thus LQQFPEL and NTQQPWQ may represent more often recognized epitope specificities whereas other sequences seem to be restricted to individual sera. Further, dominant clones may have overgrown less frequent ones during selection which may explain differences in the epitope repertoire indicated in Tables 1 and 2.

Experiments with human antisera revealed that only one of them contained antibodies reacting with the selected peptides. Nevertheless, this serum recognized four of the peptides at the same time, including LQQFPEL which was also bound by a large number of rabbit antisera, and AHMNPSP which was reactive with none of the rabbit sera in the luminescence assay. The different epitope specificity of human and rabbit antisera may be explained by different ways of immunization, i.e. gastrointestinal vs. intracutaneous, which may include different processing and presentation of antigen.

The distribution of postulated linear epitopes over gli/glu sequences shows clustering in the N-terminal part of α- and γ-type gliadins (Fig. 1). α- and γ-type gliadins contain three and four disulfide bridges, respectively, leading to the formation of loops and of a compact structure in the C-terminal part of the molecules [22] which may be less accessible to B-cell response than the N-terminal region. Thus, at least for antibodies recognizing linear epitopes or epitopes containing a linear component, the N-terminal region may possess a high antigenicity. Interestingly, short sequence stretches in the N-terminal region of α-type gliadins are suspected to be responsible for toxicity of gliadins in celiac patients [23]. Further investigations are currently being performed to examine the epitope specificity of human gliadin antibodies.

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