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Relationship between gliadin peptide structure and their effect on the fetal chick duodenum

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Beziehung zwischen der Gliadin-Peptid-Struktur und ihr Einfluß auf den fetalen Kückendarm

Zusammenfassung. Die Tendenz zur Bildung einer β-Umwandlung im α-Gliadin wurde bei Anwendung eines mathematischen Programms zur Vorhersage von B-Zelldeterminanten bestimmt, welches auf der Wahrscheinlichkeit der β-Umwandlung nach Chou und Fasman basiert. Es wurden 6 Sequenzen gefunden, die eine hohe Wahrscheinlichkeit für die Bildung von β-Umwandlungen aufwiesen. Zwischen diesen 6 Sequenzen und 3 Regionen im α-Gliadin mit der Sequenz Pro-Ser-Gln-Gln, die kürzlich als verantwortlich für die Toxizität bei Cöliakie angesehen wurden, konnte eine statistisch gesicherte Beziehung gefunden werden. Mittels Festphasensynthese wurden 7 Peptide erhalten, die die oben genannten Regionen überdeckten. Ihre Toxizität wurde im fetalen Kückendarm getestet. Die Ergebnisse weisen darauf hin, daß Peptide, welche die Sequenz Pro-Ser-Gln-Gln und Gln-Gln-Gln-Pro enthalten, an der Pathogenese der Cöliakie beteiligt sein könnten.

Summary. The tendency to form a β-turn in α-gliadin was estimated using the B-cell determinant prediction program based on the Chou and Fasman probability of β-turn formation. Six sequences possessing a high probability of β-turn formation were found. A statistically high agreement was found between these six sequences and three areas in α-gliadin with the occurrence of Pro-Ser-Gln-Gln sequence which has recently been considered responsible for toxicity in coeliac disease. By means of solid-phase synthesis seven peptides were obtained covering the above-mentioned regions. Their toxicity was tested using the fetal chick duodenum. The results support the suggestion that peptides containing the sequences Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro may be involved in the pathogenesis of coeliac disease.

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Introduction

Peptide fragments of gliadins are involved in the etiopathogenesis of coeliac disease. These peptides are generated by proteolytic cleavage during food digestion and some of them might initiate the disease in predisposed individuals. Numerous studies describe attempts to isolate and identify a specific toxic peptide. Peptide B 3142 comprising the gliadin primary sequence 3–55 has been isolated by Wieser [1, 2] using HPLC and its toxicity has been confirmed in cultivated small-intestinal biopsies. A toxic peptide marked β_{5b}-PT from β-gliadin has been prepared by Jos et al. [3].

De Ritis et al. [4] described toxicity of some peptides originating from α-gliadin after CNBr cleavage. The analysis of amino acid sequence revealed that tetrapeptide stretches Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro were present in all toxic peptides and absent in non-toxic fragments. The Pro-Ser-Gln-Gln was considered toxic also by Wieser et al. [5].

This study deals with mathematical prediction of regions in the primary structure of α-gliadin which have a high tendency to be exposed on the molecule surface. These regions could function as immunodeterminants (inducing an abnormal immune response in subjects with coeliac disease) or as binding sites important for the presumed association with the brush-border membrane. Peptides found by this prediction were synthesized and their toxicity was tested using fetal chick duodenum as an in vitro model.

Materials and methods

Synthetic peptides

The primary structure of α-gliadin was used as determined by Kasarda et al. [6] with some modifications from cDNA studies of Anderson et al. [7] and Okita et al. [8]. The tendency to form a β-turn was calculated by using a BASIC program for B-cell determinant prediction described previously [9–11]. Dodecapeptides with gliadin sequences 8–19, 45–56, 208–219 and their analogues (Table 1) were synthesized by using multiple continuous-flow solid-phase method.

Table 1. Synthetic dodecapeptides with α-gliadin sequences prepared by the continuous-flow solid-phase method

Peptide	Position	Primary structure
I	8– 19	LeuGlnProGlnAsnProSerGlnGlnGlnProGln
II	45– 56	GlnProGlnProPheProSerGlnGlnProTyrLeu
III	208–219	GlnGlySerPheArgProSerGlnGlnAsnProGln
IV	8– 19	LeuGlnProGlnAsnThrGlyGlnGlnGlnProGln
V	8– 19	LeuGlnProGlnAsnProSerAlaAlaAlaProGln
VI	8– 19	LeuGlnProGlnAsnProSerAsnAsnAsnProGln
VII	8– 19	LeuGlnProGlnAsnProSerProProProProGln

Modified residues are underlined

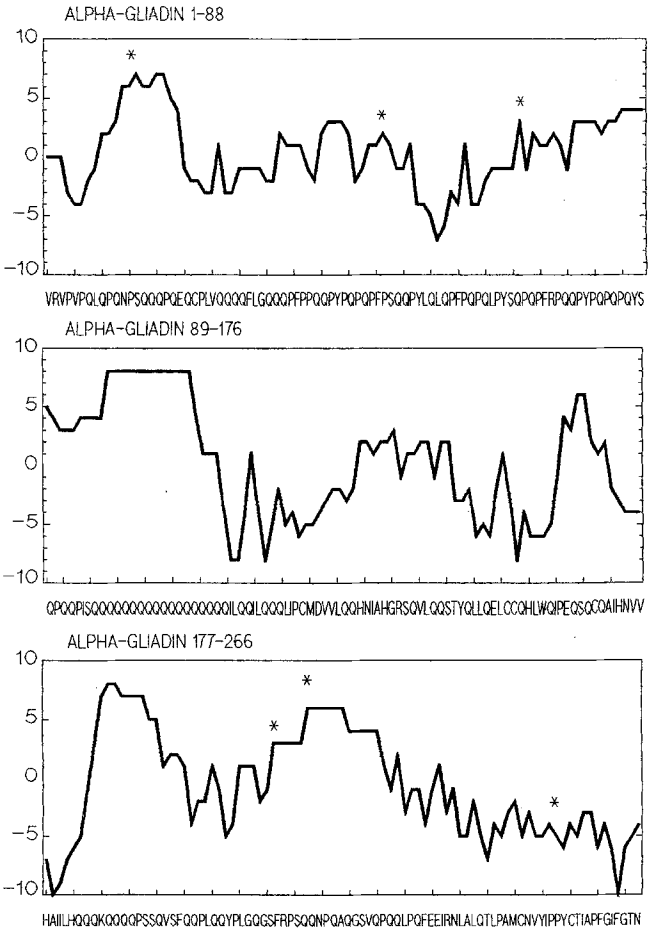

Fig. 1. Hydrophilicity profile of the whole α-gliadin primary structure calculated according to Parker coefficients in a relative scale of 0–10. β-Turns with probability $>2\times10^{-4}$ are marked by *

The synthesis was performed according to the multiple fluoren-9-ylmethoxycarbonyl/*t*-butyl (Fmoc/*t*Bu) protection strategy on the *p*-methylbenzhydrylamine resin [12]. The purity of synthetic peptides was checked by using high-performance liquid chromatography on a Separon SGX-C_{18} column and acetonitril gradient elution as described previously for natural gliadin peptides [19]. The results of HPLC analysis have been published separately [12].

Tissue culture of fetal intestine

Duodena of 12-day-old chick embryos were explanted and cultured for 2 days as described recently [13]. A modified minimal essential medium (MEM) served as culture medium. The culture was performed either in the presence of dodecapeptides (0.5 mg/ml) or in the absence of peptides (control culture). After culture the medium was removed and the tissue explants were rinsed in 0.5 ml isotonic NaCl solution and stored at −20° C. After thawing, the tissue explants were homogenized in cold isotonic NaCl solution using a Dounce microhomogenizer.

Sucrase activity determination

Sucrase activity was measured by a modification of the method of Dahlqvist [14] as described previously [15]. Protein was estimated according to Lowry [16] with bovine serum albumin as standard. Enzyme activity and protein content were related to the wet mass of tissue explants before culture. Sucrase activity was expressed as a percentage of the increase in activity during a control culture. Protein was given as a percentage of the value measured in the duodenal explants after control culture. All results were expressed as averages and standard error of the means. One-tailed Student's *t*-test for paired samples (SPSS/PC+Statistic software package) was used to evaluate a statistical significance of the inhibition.

Results

The sequence of 266 amino acids of α-gliadin reported by Kasarda was subjected to computer analysis using our program for B-cell determinant prediction. Six tetrapeptides having β-turn occurrence probability greater than 2×10^{-4} were found (Table 2).

The local hydrophilicity of each heptapeptide segment was calculated using Parker's et al. [17] hydrophilicity parameters. The hydrophilicity profile (Fig. 1) demonstrates positions of these β-turns, which overlap all three areas with Pro-Ser-Gln-Gln sequences and coincide with three of four amino acid residues Pro-Ser-Gln. The probability of this phenomenon calculated from combinatorial variants was found at 3.66×10^{-6}, which confirms the high statistical significance of the agreement observed.

Table 2. Predicted β-turn occurrence in α-gliadin

The first column gives the sequence number of the first amino acid; *P*, the probability of β-turn occurrence $\times 10^{-4}$. The conformational potentials of α-helix, β-turn, and β-sheet are given, followed by the mean hydrophilicity for heptapeptide fragments centered at corner positions of a β-turn

No.	β-Turn sequence	P	Potential of			Hydrophilicity	
			α-Helix	β-Turn	β-Sheet		
12	Asn-Pro-Ser-Gln	5.94	0.78	1.37	0.82	5.10	5.66
49	Phe-Pro-Ser-Gln	2.18	0.89	1.13	0.95	2.79	2.23
66	Leu-Pro-Tyr-Ser	2.22	0.81	1.17	1.02	1.66	1.66
207	Gly-Gln-Gly-Ser	2.01	0.75	1.38	0.84	1.09	1.39
212	Arg-Pro-Ser-Gln	2.58	0.86	1.22	0.83	3.16	3.23
252	Pro-Pro-Tyr-Cys	4.48	0.63	1.34	0.94	−0.14	−1.01

The synthetic preptides I, II, and III comprising the sequences 8–19, 45–56 and 208–219 of α-gliadin inhibited the increase in sucrase activity in cultured fetal intestine explants (Table 3). The effect of peptide I was most pronounced and was not lost when Pro-Ser in positions 14–15 were replaced by Thr-Gly (peptide IV). This substitution was used to confirm the anticipation of β-turn for toxicity because the tetrapeptide Asn-Thr-Gly-Gln has the highest possible probability to form a β-turn without a Pro residue. If however, the three Gln in positions 16–18 were substituted with three Ala (peptide V), the increase of sucrase acitivity was not inhibited at all. The inibition by peptides I and II–IV is statistically significant at $p < 0.05$ and 0.01 respectively.

The next two modifications were designed to confirm the necessity of the presence of the Gln-Gln-Gln fragment. Exchange of Ala-Ala-Ala eliminates the effect of side-chains on glutamines, Asn-Asn-Asn mimicked the glutamine side-chain with shortened length (peptide VI) and Pro-Pro-Pro (peptide VII) affected the backbone conformation. Only the peptide with three glutamines inhibited the increase of sucrase activity on fetal chick duodenum during short-time culture to 59%, while the other three peptides (V, VI, and VII) were not active. The inhibition by peptides II and III with only Pro-Ser-Gln-Gln but without Gln-Gln-Gln-Pro was smaller than that exerted by peptide I containing both sequences.

Discussion

The pathogenesis of coeliac disease has not so far been clarified. Numerous studies describe attempts to isolate a specific toxic peptides from wheat proteins. Our previous study was concerned with isolation of the glycopeptide Glyc-Gli [18] and some peptide fragments of the digested α-gliadin by using HPLC [19].

Any biological activity of proteins or peptides is closely related to their spacial arrangement. A protein backbone can form three different ordered secondary structures, the α-helix, the β-sheet and the reverse turn (β-turn). In a β-turn backbone the overall direction is reversed and loops are formed responsible for globular shape of proteins. The β-turn residues are very seldom buried in the interior of the protein but tend to protrude from the protein surface.

The computer prediction model, supplemented by protein hydrophilicity profiles, has been applied to the primary structure of α-gliadin. We found a statistically highly significant agreement between the six predicted sequences of β-turns and three areas with the occurrence of the Pro-Ser-Gln-Gln sequence, suggested to be responsible for toxicity in coeliac disease by De Ritis et al. [4] and Wieser et al. [5]. In the primary structure of α-gliadin this sequence occurs in positions 13–16, 50–53, and 213–216 (Table 4). The stretch 206 to 217 is, in addition, identical in eight of twelve amino acid residues with the sequence 384–395 of the adenovirus protein Ad12 E1b, which may have a relation to the pathogenesis of coeliac disease [20, 21]. The sequence Pro-Ser-Gln-Gln in position 213–216 is 75% identical with the Arg-Pro-Ser-Gln-Cys sequence 390–394 of the adenovirus protein, while the predicted fragment is 100% identical. The tetrapeptide Phe-Pro-Ser-Gln in position 49–52 likewise overlaps with de Ri-

Table 3. Sucrase activity and protein contents in cultured fetal chick duodenum in presence of synthetic dodecapeptides

Peptide	Sucrase activity		Protein in explants
	In explants	In medium	
I	59* ±18 (4)	92±14 (3)	101± 8 (4)
II	70** ±10 (4)	141± 6 (3)	105± 7 (4)
III	74** ± 9 (7)	119±15 (7)	105± 5 (6)
IV	53** ± 8 (5)	100±14 (5)	97±11 (5)
V	103 ±15 (9)	135±12 (9)	105± 5 (9)
VI	107 ±17 (8)	128±14 (7)	102± 6 (8)
VII	110 ±15 (8)	125±13 (8)	104± 4 (8)

Results are expressed as percentage increase relative to control culture. Mean sucrase activity before culture was 76 nmol min^{-1} g^{-1}, after control culture in tissue explants 475 nmol min^{-1} g^{-1} and in culture medium 285 nmol min^{-1} g^{-1}. Mean protein content after control culture was 81 mg/g wet tissue. Number of experiments in parentheses, significance $P < 0.05$ (*), $P < 0.01$ (**)

Table 4. Primary sequence of α-gliadin (part), β-casomorphin and E1b protein of adenovirus Ad12 (part)

Peptide	Sequence										
α-Gliadin	Pro^{10}	Gln	Asn	**Pro**	**Ser**	**Gln**	**Gln**	Gln	Pro	Gln	
	Glu	Gln	Val	Pro	Leu	Val	Gln	Gln	Gln	Gln	
	Phe	Leu	Gly	Gln	Gln	Gln	Pro	Phe	Pro	Pro	
	Gln	Gln^{41}									
α-Gliadin	Pro^{42}	*Tyr*	*Pro*	Gln	*Pro*	Gln	*Pro*	Phe	**Pro**	**Ser**	**Gln**
	Gln	Pro	Tyr	Leu	Gln						
β-Casomorphin		*Tyr*	*Pro*	Phe	*Pro*	Gly	*Pro*	Ile			
α-Gliadin	Gln^{203}	Tyr	Pro	*Leu*	Gly	Gln	*Gly*	Ser	*Phe*		
	Arg	***Pro***	***Ser***	***Gln***	**Gln**	*Asn*	Pro				
				Leu^{384}		Arg	Arg	*Gly*	Met	*Phe*	
	Arg	*Pro*	*Ser*	*Gln*	Cys	*Asn*					

Identical residues are in italics, β-turns are underlined and De Ritis's sequence Pro-Ser-Gln-Gln is in boldface

tis's sequence in 75% and is closely related to α-gliadorphin heptapeptide Tyr-Pro-Gln-Pro-Gln-Pro-Phe (positions 43–49, identical in five of seven amino acids with β-casomorphin), which affects the stimulation of lymphocytes in patients with coeliac disease [22]. The opioid-like receptor for this heptapeptide on peripheral blood lymphocytes has been described by Graf et al. [23].

Gliadin peptides added to the culture medium inhibit the biosynthesis of duodenal sucrase [24] with missing increase of enzymatic activity. This event seems to be specific, since several non-gliadin proteins or peptides (ovalbumin, bovine serum albumin, pepsin/trypsin-digested casein) had no inhibitory effect [15]. These results suggest that fetal intestine may represent a suitable in vitro model for the investigation of gliadin toxicity. According to Auricchio et al. [25], undifferentiated enterocytes are a prerequisite for the effect of gliadin, which is thought to represent a direct interaction not mediated by cells of the immune system.

De Ritis et al. [4] suggested that the toxicity of gliadin is mainly due to two tetrapeptide sequences Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro. Synthetic dodecapeptides tested in this study are similar to α-gliadin sequences 8–19, 45–56, and 208–219, and contain the presumably toxic tetrapeptide Pro-Ser-Gln-Gln. These sequences overlap with β-turns predicted by computer modelling. Peptide I contains additionally the sequence Gln-Gln-Gln-Pro, overlapping the former. Indeed all three dodecapeptides inhibited the increase in sucrase activity in fetal chick duodenum with statistically significance. Their effectiveness, however, was different. The highest inhibition was observed with peptide I, and the lowest with peptide III, which is similar to the E1b protein of adenovirus Ad12. The strong effect of peptide I might be attributed to the existence of both tetrapeptide sequences, whereas the other two peptides (II and III) contain only one Pro-Ser-Gln-Gln sequence. A different statistical significance of this inhibition was affected by a smaller number of culture experiments in some cases. Interestingly, only the substitution of Gln-Gln-Gln (but not of Pro-Ser) eliminate the toxicity of peptide I, whereas the exchange of Pro-Ser for Thr-Gly in peptide I (an exchange which is supposed not to influence the β-turn structure) did not diminish the effect of this peptide at all, the substitution of the three glutamines eliminated the toxicity of peptide I completely. This may be explained by the fact that these three residues are part of both the overlapping and presumably toxic tetrapeptide sequences. Our results are in agreement with the suggestion of Auricchio et al. [25] that the sequence Gln-Gln-Gln-Pro, when part of a larger peptide, may be toxic to the atrophic coeliac mucosa, in vitro.

The sequence Pro-Ser-Gln-Gln does not occur in proteins like caseins, α-lactalbumin, β-lactoglobulin or zein, which do not cause coeliac disease [5]. Our results support the opinion of De Ritis et al. [4] on the significance of the Pro-Ser-Gln-Gln and Gln-Gln-Gln-Pro for the toxicity of gliadin peptides in coeliac disease. We suggest that these sequences may be important with respect to (a) the occurrence of high-probability β-turns in all three areas with recurrence of the De Ritis's tetrapeptide, (b) high levels of hydrophilicity in fragments Asn-Pro-Ser-Gln and, in particular, Arg-Pro-Ser-Gln, similar to the sequence of the adenovirus protein Ad12 E1b.

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