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β Structure motif recognition by anti-gliadin antibodies in coeliac disease

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Abstract A 20-amino acid synthetic peptide from the N-terminal region of γ3 avenin yields a surprisingly strong reactivity with anti-gliadin antibodies (AGA) of coeliac sera, comparable to that of a gliadin extract. In contrast, a low reactivity is observed with five similar peptides derived from α-gliadin, γ70 and ω1 secalins. Circular dichroism studies of these peptides show that the avenin peptide displays the highest β-turn content (30%), while other peptides yield much lower values. In agreement with circular dichroism data, nuclear magnetic resonance data point to the presence of a β-turn in the avenin peptide DPSEQ segment, a sequence with a high statistical β-turn preference. A strong linear dependence between AGA reactivity and β-turn content was observed for these peptides, indicating for the first time a role of β-turn motifs in anti-gliadin antibodies recognition in coeliac disease. This suggests that circulating AGA in coeliac patients comprise not only linear but also conformational antibodies against β-turn motifs. Polyclonal antibodies raised against the avenin peptide containing β-turn motifs react by immunoblotting with all gliadin, hordein and secalin proteins, which are rich in β-turn conformations, despite that their primary structures are unrelated to that of the peptide.

Key words: β-turn motif; Coeliac disease; Anti-gliadin antibody

1. Introduction

Coeliac disease (CD) is provoked by the ingestion, by genetically susceptible individuals, of toxic gluten proteins, the so-called gliadins, hordeins, secalins and avenins, present in wheat, barley, rye and oats [1]. An abnormally high level of circulating anti-gliadin antibodies (AGA) in serum is the most relevant characteristic of the humoral immune response in untreated coeliac patients following ingestion of toxic gluten [2]. Although anti-gliadin antibodies cross-react with all these gluten proteins, the specific epitopes capable of raising such antibodies have not yet been determined. Immune cross-reactivity can be ascribed not only to conserved specific linear sequence motifs, but also, as suggested, to the existence of β-turn conformational motifs involving potentially toxic sequences located at the N-terminal region of α-gliadins [3]. Nevertheless, the cross-reactive recognition of β-turn motifs by anti-gliadin antibodies has not yet been addressed. The present manuscript describes the first evidence of the implication of secondary structures in anti-gliadin antibody recognition in coeliac sera, showing a linear relationship between the β-turn content of antigens and IgA-AGA levels.

2. Materials and methods

2.1. Synthesis of peptides from cereal proteins

Twenty amino acid synthetic peptides derived from the N-terminal region of γ3-avenin, α-gliadin, γ70 and ω1-secalin were synthesized on an automated multiple peptide synthesizer (AMS 422, Abimed) using solid-phase/Fmoc-chemistry [4]. Purity of the peptides was confirmed by RP-HPLC and MALDI-TOFF mass spectrometry.

2.2. Rabbit immunization and polyclonal antibodies generation

New Zealand rabbits were immunized with 250 μg of each KLH-coupled synthetic peptide, using complete Freund's adjuvant for the first immunization. Two muscular boosts of 150 μg of the same material with incomplete Freund's adjuvant were given 4 and 7 weeks later. Sera were collected 7–10 days after the last injection. The polyclonal antibody against the γ3 avenin(1–20) peptide was purified from sera by affinity chromatography by using a protein A-Sepharose column.

2.3. Ethanol extraction of cereal prolamins

Gliadins, hordeins, secalins, avenins and zeins where extracted from wheat, barley, rye, oats and maize flours, respectively, using 60% (v/v) aqueous ethanol as described elsewhere.

2.4. IgA-AGA determination by ELISA

Polystyrene microtitre plates were coated overnight at 4°C with 100 μl/well of synthetic peptides and cereal extracts, dissolved in 0.1 M carbonate buffer, pH 9.6, to a concentration of 10 μg/ml. Non-specific binding sites were blocked with 2% BSA in PBS-T. Plates were then incubated with human sera, 1:100 diluted in PBS, from coeliac and healthy patients for 1 h at 37°C. This was followed by further incubation with goat anti-human IgA conjugated to alkaline phosphatase for 1 h at 37°C. Color reactions were developed by adding p-NPP substrate and the absorbance was read at 405 nm.

2.5. ELISA for cereal extracts

Polyclonal antibodies generated against synthetic peptides were used to test wheat, barley, rye and oats extracts. The ELISA was nearly identical to the above described except that anti-rabbit IgG coupled to alkaline phosphatase was used instead of anti-human IgA.

2.6. Immunoblotting

After one-dimensional SDS-PAGE, protein extracts from wheat, barley, rye and oat were transferred onto PVDF membranes. The blots were developed either with a coeliac serum (1:50) incubated with alkaline phosphatase-conjugated anti-human IgA or with rabbit polyclonal IgG (10 μg/ml) against γ3-avenin incubated with alkaline phosphatase-conjugated anti-rabbit IgG. The blots were developed with BCIP.

2.7. Patients

Fifty healthy sera and fifty biopsy-proved CD sera were tested by ELISA. Children were on a gluten-containing diet when serum samples were obtained.

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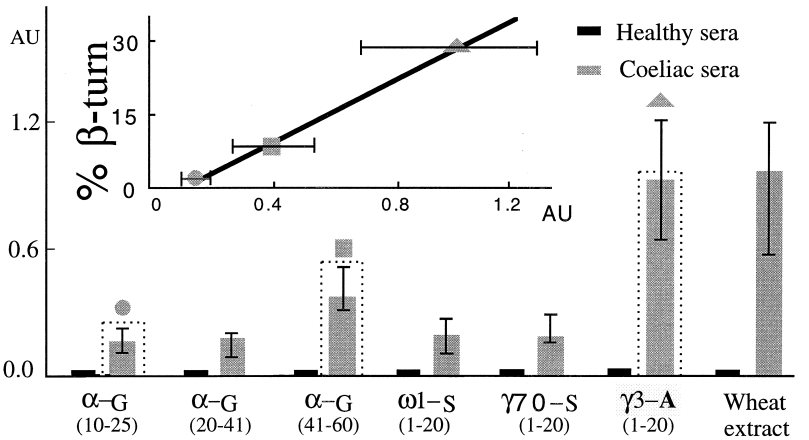

Fig. 1. Comparison of IgA-AGA levels by ELISA of coeliac sera as revealed by six synthetic peptides and a wheat extract. AGA levels are mean values from a total of six coeliac and healthy sera. Correlation between β-turn content of three selected peptides and AGA detection is inserted. G, gliadin; S, secalin; and A, avenin.

2.8. Circular dichroism studies

The secondary structure was studied by circular dichroism as previously described [5]. 50-μg aliquots of the peptides were resuspended in 5 mM Tris-HCl, pH 7.4 to give a concentration of 0.14 mg/ml and their far-UV spectra recorded in a Jasco Spectropolarimeter model J-720 at 0.2-nm intervals over the 190–250-nm wavelength range at room temperature. A 0.1-cm pathlength cell was employed to accumulate 20 scans. The percentages of different secondary structure motifs were obtained by analysis of the circular dichroism spectra using the Lincomb algorithm [6].

2.9. ^{1}H-NMR spectra

Peptide concentration for NMR experiments were 5 mM in 0.5 ml of H_2O/D_2O (9:1 ratio by volume) or D_2O. pH values used were 2.5, 3.0, 4.0 and 5.0 (not corrected for isotope effects). Sodium (3-trimethylsilyl-*2,2,3,3-^2H*)propionate (TSP) was used as an internal reference. The ^{1}H-NMR spectra were acquired on a Bruker AMX-600 pulse spectrometer at 5°C, 10°C and 25°C. Phase sensitive two-dimensional total correlated spectroscopy (TOCSY) [7], nuclear Overhauser enhancement spectroscopy (NOESY) [8], and rotating frame nuclear Overhauser effect spectroscopy (ROESY) [9] spectra were recorded by standard techniques using presaturation of the water signal and the time-proportional phase incrementation mode [10]. Mixing times of 80 and 150 ms were used for NOESY and 200 ms for NOESY spectra. Acquisition data matrices were defined by 2018×512 points in t_2 and t_1, respectively, and zero-filled to a $4K \times 2K$ complex matrix prior to Fourier transformation.

3. Results

3.1. Measurements of anti-gliadin antibody levels in sera by ELISA using cereal synthetic peptides as antigens vs. wheat gliadin extracts

Total gliadin extracts or purified gliadin fractions from wheat are routinely used as coating antigens by ELISA to determine AGA levels. We investigated the possibility of using smaller polypeptides from gliadins, secalins and avenins as antigens for CD screening by ELISA.

Six 20-amino acid synthetic peptides derived from the N-terminal region of α-gliadin, γ35 and ω1 secalins and γ3 avenin, all containing the potentially toxic motifs QQQP, QQQQ, PSQQ and PQQP [11,12], together with a crude gliadin extract, were evaluated against coeliac sera by ELISA. The avenin peptide yields the highest IgA-AGA values (Fig. 1, top), nearly identical to those of the gliadin extract. Conversely, other peptides tested show a much lower response, except for the wheat gliadin peptide α(41–60), which displays intermediate values. This high immunoreactivity of both the avenin peptide and the wheat gliadin extract was confirmed employing at least 50 coeliac sera (data not shown). These high levels were extensively reduced in both cases when sera were previously incubated with either the avenin peptide or the gliadin extract (data not shown), suggesting that the two antigens are recognizing the same type of anti-gliadin antibodies. Their primary structures alone cannot be responsible for this comparable immunoreactivity since the avenin peptide and gliadins exhibit only a low degree of identity. In fact, only short sequences comprising three to four amino acids of the avenin peptide, corresponding to residues QPYP, QQPF and DPS, were found in the polypeptide chain of γ-gliadin and QQQP in the low molecular weight glutenin. A similar low degree of sequence identity was found in hordeins and secalins [13].

3.2. Secondary structure of synthetic peptides

To investigate the role of peptide conformation on the differences in response to CD between avenin and other synthetic peptides, circular dichroism studies were carried out. Circular dichroism spectra (Fig. 2) and corresponding secondary structure analysis indicate marked differences in the β-turn content of the peptides. The avenin peptide had the highest levels of β-turn (30%) while the α gliadin(41–60) peptide showed an intermediate content (9% β-turn) and the other gliadin or secalin peptides much lower values (Table 1). By comparing these data with sera levels, a direct relationship

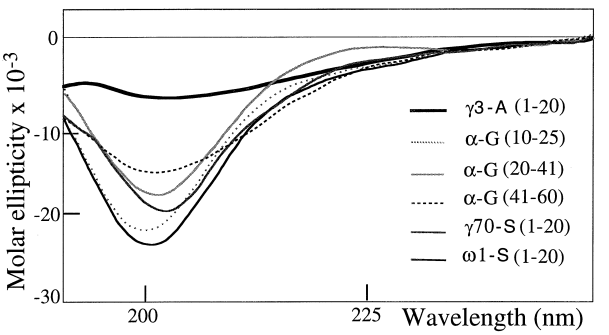

Fig. 2. Circular dichroism assays. Results are expressed in terms of molar ellipticity in units of deg cm^2 dmol^{-1}.

Table 1
Secondary structure obtained by the analysis of the circular dichroism spectra (left) and amino acid sequences (right) of six different avenin, gliadin and secalin synthetic peptides

Peptides	Secondary structure				Synthetic peptide sequence
	α-helix	β-sheet	β-turn	Random	
γ3-A (1-20)	2.3	22.0	**30.0**	46.0	V Q Y D P S E Q Y - Q - P Y P E **Q Q Q P** F
α-G (10-25)	0.0	0.1	1.3	99.0	P Q N - P S **Q Q - -Q P** P E Q V P L V
α-G (20-41)	1.0	4.4	6.2	88.0	E Q V - P L V Q Q - Q F P G **Q Q Q Q** F P P Q Q
α-G (41-60)	1.7	11.0	9.0	78.0	Q P Y P Q P Q P F- - **P S Q Q** P Y L Q L Q
γ70-S (1-20)	1.2	1.0	5.3	93.0	M Q V N P S G Q V - Q P P **Q Q Q P** F P Q
ω1-S (1-20)	0.0	0.0	1.0	99.0	R Q L N P S E Q E L Q S **P Q Q P** V P G

The percentage of secondary structure from Fig. 2 was calculated by the Lincomb algorithm [6]. Conserved sequences are indicated by shaded boxes. Gaps are included to achieve maximal sequence identity. The potentially toxic tetrapeptide motifs are labeled.

between IgA-AGA response and β-turn content became evident (Fig. 1, top, insert). This result suggests for the first time a role of β-turn motifs in the AGA recognition in CD.

3.3. *Massive immunodetection of gliadins, hordeins, secalins and avenins by a polyclonal antibody against avenin peptide*

In a direct ELISA the polyclonal antibody of the avenin peptide nearly recognizes to the same extent the four different gliadin, hordein, secalin and avenin extracts (Fig. 3, top, left). Conversely, polyclonal antibodies generated against peptides derived from gliadins and secalins displayed quite different values with these four cereals extracts (Fig. 3). The fact that this antibody against the avenin peptide yields a high immune response to these four cereal extracts, with which it has in common secondary rather than primary structure similarities, suggests a role of β-turn motifs.

These results are also supported by immunoblotting analy-

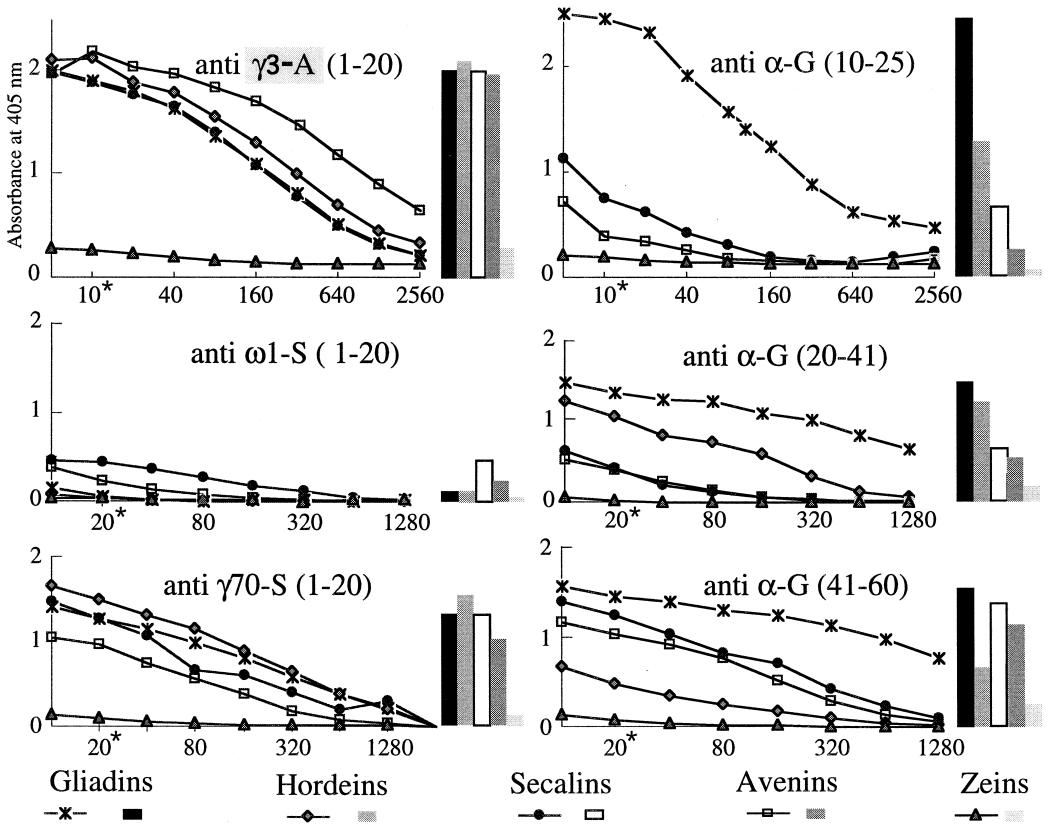

Fig. 3. Recognition by ELISA of gliadins, hordeins, secalins, avenins and zeins employing polyclonal antibodies against synthetic peptides. Values of a single dilution indicated by an asterisk are displayed by bars. Dilution values in abscissae are in thousands.

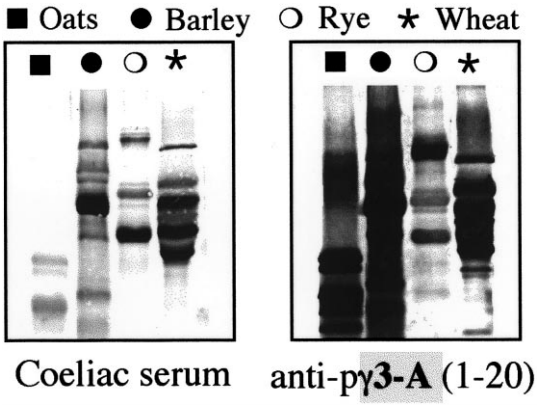

Fig. 4. Immunoblotting of wheat, barley, rye and oats alcohol extract against a coeliac serum (left) and the polyclonal antibody against the avenin peptide (right). 40 μg of extracts were loaded. Blots were developed with a coeliac serum diluted 1:50 and polyclonal anti-γ3-A antibody at 10 μg/ml, respectively.

sis, which show that all protein components of gliadins, hordeins, secalins and avenins strongly immunoreact with the polyclonal antibody against the avenin peptide (Fig. 4, right). The immunoblot pattern is nearly identical to that obtained when using coeliac sera (Fig. 4, left) instead of the same four cereal extracts. Data support that both human AGA and rabbit AGA generated against the avenin peptide recognize the same cereal protein components, thus once more suggesting that β-turn motifs, and not only the primary structure, are involved in this immune recognition process.

3.4. *^{1}H-NMR conformational studies of the avenin peptide*

To gain further insight at the residue level about the structural requirements of the avenin peptide (VQYDPSEQYQPY-PEQQQPF) its conformational preferences in solution were studied by ^{1}H-NMR. It is interesting to point out that the spectra of the avenin peptide show a very small dispersion of chemical shifts of non-labile protons due to the repetitive amino acid sequence (E2, E7, E10, E15, E16, E17; Q8, Q14; P5, P11, P13, P18) with nearly identical chemical shift values. In spite of that, the corresponding ^{1}H-NMR spectra at various pH values could be assigned using the standard sequential assignment procedure [14], and at least one sequential NOE was detected for each amino acid. Although the observed data are not sufficient to determine a three-dimensional structure, the spectroscopic parameters strongly suggest the presence of non-random conformations at the N-terminus. Evidence for a local turn structure centered on P5-S6 was provided by the NOE pattern, NOE intensities and the amide shift temperature dependence (Fig. 5, left). Thus, the observation of (1) specific relatively intense NOE cross-correlations between adjacent amide protons, S6-Q7 and Q7-E8 (Fig. 5, right); (2) non-sequential (*i*, *i*+2; *i*, *i*+3) dipolar interactions, correlating S6 and Q7 NHs to D4 H_β (data not shown); and (3) low H_N temperature coefficients, 3.0 ppb/K and 2.5 ppb/K of S6 and Q7, respectively, is only compatible with the presence of a population of β-turn-like [15] structures at residues D4-P5-S6-E7-Q8 of the avenin peptide sequence.

4. Discussion

One of the main questions still remaining to be addressed concerning the humoral immune response in coeliac disease is the precise type of specific epitopes in gluten components capable of generating anti-gliadin antibodies. As has been suggested [3], the immunoreactivity of anti-gliadin antibodies against gliadins could be ascribed not only to specific linear sequence motifs, but also to the existence of β-turn conformational motifs which have been demonstrated to occur in gliadins, mainly in the N-terminal region. However, no experimental data on this question had been reported to date.

We designed seven 20-amino-acid synthetic peptides derived from the coeliac-activating N-terminal regions of α-gliadin, γ70 and ω1 secalin and γ3 avenin containing at least one of the potentially toxic tetrapeptide motifs QQQP, QQQQ, PSQQ and PQQP. These peptides show a high degree of similarity and a varying content of secondary structure (1–30% β-turn), as revealed by circular dichroism. Peptides were assayed as antigens in an IgA-AGA ELISA against coeliac sera using a gliadin extract and non-coeliac sera as controls. The avenin peptide with the highest β-turn content yielded the highest IgA-AGA values, nearly identical to those of the gliadin ex-

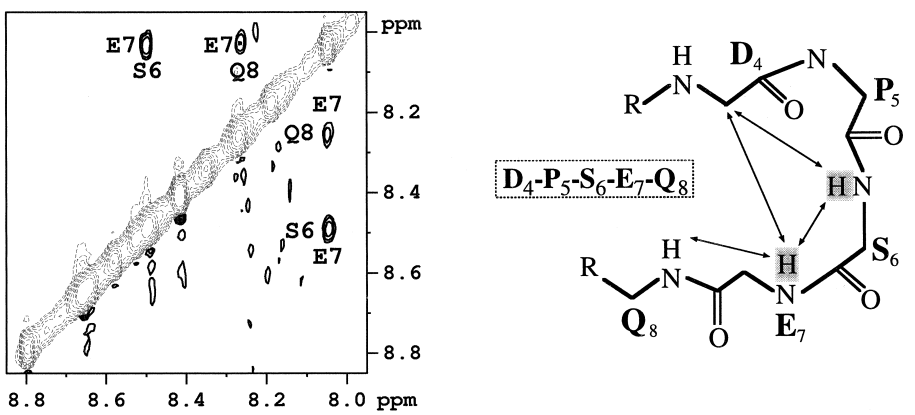

Fig. 5. Left: Schematic drawing compatible with NMR data of the β-turn structure adopted by residues D4-P5-S6-E7-Q8 of γ3-A(1–20) in solution. Detected strong H_N-H_N and H_β-H_N (*i*, *i*+2; *i*, *i*+3) NOEs are indicated by arrows. H_N protons with low temperature coefficients (<3.0 ppb/K) are dashed. Right: H_N H_N NH-NH region of ROESY spectra of γ3-A(1–20). Experimental conditions were 5 mM H_2O/D_2O 9:1, pH 5.0, 5°C and 200 ms mixing time. Positive (cross peaks) and negative (diagonal peaks) signals are shown as dashed and continuous contour lines, respectively. Only labeled sequential H_N-H_N cross peaks corresponding to S6-E7 and E7-Q8 appear in all experimental conditions used (pH: 2.5, 3.0, 4.0 and 5.0; temperature: 5°C, 10°C and 25°C; NOESY: 80 ms and 150 ms, and ROESY: 200 ms mixing time).

tract, while other synthetic peptides yielded either moderate or much lower response. Our results clearly support that the recognition of this type of peptides by anti-gliadin antibodies depends on their β-turn content, and that the role of their primary structure is less significant. Due to the high immunoreactivity of the avenin peptide with coeliac sera, its potential applicability as a coating antigen for CD screening as an alternative to gliadin-based ELISA [16] is currently under study.

It is well known that in short peptides a rigorous interpretation of NOE intensities in geometric structural terms is not possible due to the presence of multiple conformations interconverting in a rapid equilibrium. However, in cases where appreciable population of structures exists, it is possible to discuss the preferred peptide structure on the basis of the experimental NMR data. Thus, in agreement with circular dichroism data, the conformational preferences of γ3-A in solution determined in this work fit closely with a β-turn-like structure at residues D4-P5-S6-E7-Q8 as has been illustrated in Fig. 5.

ELISA data show that polyclonal antibodies raised against the avenin peptide, which contains the β-turn conformational preference, clearly recognize to the same extent gliadins, hordeins, secalin and avenins, while those raised against the ω1 secalin peptide, containing a tetrapeptide similar sequence (N4-P5-S6-E7-Q8), but no β-turn preference (see circular dichroism data, Table 1), does not. This strongly suggests that antibody recognition is based on a structural β-turn requirement. Recently, extensive work on the analysis and conformational properties of β-turns has been performed to gain insight into the factors responsible for the formation and stability of this type of structure. Thus, the type dependent positional potential for each amino acid at each of the 4 positions (*i*, *i*+1, *i*+2, *i*+3, *i*+4) of turns has been determined [17]. For a type I turn conformation, the most likely in γ3 avenin and ω1 secalin, both peptides contain amino acids with high statistical β-turn preferences in XPSEQ (X=Asn or Asp). However, given that there is no appreciable turn content in the ω1 secalin peptide, some other specific interactions could account for the stability of the avenin peptide structure. In this regard, the hydrophobic interactions of the amino acids adjacent to the turn should play an important role in the stability of the β-turn motif of the avenin peptide.

The role of β-turn conformation was further studied by attempting to characterize conformational antibodies capable of identifying β-turn epitopes in gliadins, hordeins, secalins and avenins. Rabbit polyclonal antibodies against the avenin peptide, which contains the highest percentage of β-turn, clearly recognize all these protein components from the four cereals in ELISA and immunoblotting analyses.

This massive immune recognition may be attributed predominantly to conformational antibodies against β-turn conformations, given that the avenin peptide and gliadins, hordeins and secalins have no primary structure similarities. In the case of avenins, whose sequences comprise that of the peptide, recognition could be ascribed to linear and/or conformational antibodies. In the same way that rabbit AGA, generated by a single short peptide, recognizes β-turn containing gliadins, hordeins, secalins and avenins, circulating AGA in coeliac sera recognize not only linear but also conformational epitopes. Likewise, human AGA can in principle cross-react either with large molecules containing both specific linear or β-turn motifs, or with peptides showing a low degree of sequence identity, provided they are rich in β-turn moiety. This could explain why all coeliac sera react with gliadins, hordeins [18–20], secalins [21] and avenins [22] regardless whether the coeliac patient has consumed wheat, barley, rye or oats in the diet.

These results encourage to extend the study of the implications of secondary structure in coeliac disease to other aspects like pathogenesis, e.g. whether toxic motifs are more likely to be conformational β-turn structures rather than the conventionally-assumed linear tetrapeptide moieties or structures combining β-turn and linear motifs since both are mixed up in the coeliac-activating N-terminal region.

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References

[1] Marsh, M.N. (1992) Gastroenterology 102, 330–354.
[2] Walker-Smith, J.A., Guandalini, S., Schmitz, J., Shmerling, D.H. and Visakorpi, J.K. (1990) Arch. Dis. Child. 65, 909–911.
[3] Tatham, A.S., Marsh, M.N., Wieser, H. and Shewry, P. (1990) Biochem. J. 270, 313–318.
[4] Gausepohl, H., Boulin, C., Kraft, M. and Frank, R.W. (1992) Peptide Res. 5, 315–320.
[5] Soto, C. and Castaño, E.M. (1996) Biochem. J. 314, 701–707.
[6] Perczel, A., Park, K. and Fasman, G.D. (1992) Anal. Biochem. 203, 83–93.
[7] Bax, A. and Davies, D.G. (1985) J. Magn. Reson. 65, 355–360.
[8] Kumar, A., Ernst, R.R. and Wütrich (1980) Biochem. Biophys. Res. Commun. 95, 1–6.
[9] Bothner-By, A.A., Stephens, R.L., Lee, J.M., Warren, C.D. and Jeanloz, R.W.J. (1984) Am. Chem. Soc. 106, 811–813.
[10] Marion, D. and Wütrich, K. (1983) Biochem. Biophys. Res. Commun. 113, 967–974.
[11] Ellis, H.J., Doyle, A.P., Sturgess, R.P. and Ciclitira, P.J. (1992) Gut 33, 1504–1507.
[12] Weiser, H. and Belitz, H.D. (1992) Z. Lebensm. Unters. Forsch. 195, 22–26.
[13] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[14] Wütrich, K. (1986) John Wiley, New York, NY.
[15] Dyson, H.J., Rance, M., Houghten, R.A., Lerner, R.A. and Wright, P.E. (1988) J. Mol. Biol. 201, 161–200.
[16] Alfonso, P., Escobar, H. and Méndez, E. (1997) J. Pediatr. Gastroenterol. Nutr. 24, 465.
[17] Hutchinson, E.G. and Thornton, J.M. (1994) Prot. Sci. 3, 2207–2216.
[18] Rocher, A., Soriano, F., Molina, E., González-Limas, G. and Méndez, E. (1995) Biochim. Biophys. Acta 1247, 143–148.
[19] Hekkens, J.M. and Van Twist-de Graaf, M. (1990) in: Serologic Diagnosis of Coeliac Disease (Chorzelski, T.L., Beutner, E.H., Kumar, V. and Zalewski, T.K., Eds.) Ch. 2, pp. 21–57, CRC Press, Boca Raton, FL.
[20] Friis, S.U., Noren, O., Sjostrom, H. and Gudmand-Hoyer, E. (1986) Clin. Chim. Acta 155, 133–142.
[21] Rocher, A., Calero, M., Soriano, F. and Méndez, E. (1996) Biochim. Biophys. Acta 1295, 13–22.
[22] Rocher, A., Colilla, F., Ortiz, M.L. and Méndez, E. (1992) FEBS Lett. 310, 37–40.