Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 2039–2044

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

INCREASED HLA-DQ2-AFFINITY OF A SYNTHETIC GLIADIN PEPTIDE BY ACID-INDUCED DEAMIDATION OF GLUTAMINE RESIDUES

Christian Terreaux, Tilmann Walk, Yvonne van de Wal[a], Frits Koning[a], Günther Jung and Burkhard Fleckenstein*

Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany and [a]Department of Immunohematology and Bloodbank, Leiden University Hospital, Albinusdreef 2, NL-2333 ZA Leiden, The Netherlands

Received 23 April 1998; accepted 1 July 1998

Abstract
Presentation of antigenic gliadin peptides by the HLA-DQ2 molecule is considered as a key event in celiac disease pathogenesis. Chemical deamidation of the side chains of glutamine residues might have a strong influence on gliadin peptide binding to the DQ2 molecule. Glutamine deamidation of A-gliadin peptide (45-56) under acidic conditions corresponding to the gastric environment was studied using RP-HPLC, Edman degradation, capillary electrophoresis and electrospray mass spectrometry. Deamidation resulted in peptides with increased DQ2-affinities as assessed in a cell-free binding assay.

Celiac disease (CD) or gluten sensitive enteropathy is characterized by a typical flat small intestinal mucosa and is caused by the ingestion of the protein part of wheat or related cereals, leading to malabsorption and growth abnormalities. Although the events leading to mucosal changes in CD remain more or less unclear, some lines of evidence are contributing towards a single major hypothesis. First, gliadin, the alcohol-soluble fraction of gluten, induces the toxic effects in CD and remission can be obtained on a gluten-free diet[1]. Second, CD is strongly associated with the expression of the HLA-DQ(α1*0501,β1*0201) molecule (DQ2), encoded either in *cis* (in HLA-DR3 patients) or in *trans* (in HLA-DR5, -DR7 heterozygous patients)[2,3]. Finally, gliadin-specific HLA-DQ2 restricted T cells have been isolated from the intestinal mucosa of celiac patients[4]. Thus, presentation of gliadin peptides by HLA-DQ2 molecules and subsequent T cell activation are most probably key events in celiac disease pathogenesis. In contrast, previous reports on the binding of gliadin peptides to the CD-associated DQ2 molecule[5,6] showed only low or moderate affinities for these peptides.

Fax: +49 / 7071 / 29-5560; e-mail: burkhard.fleckenstein@uni-tuebingen.de

0960-894X/98/$ - see front matter
PII: S0960-894X(98)00357-6

Peptide binding studies with the HLA-DQ2 molecule[7,8] have indicated that negatively charged residues in peptide ligands can significantly increase their affinity to this allele. Therefore, hydrolysis of glutamine residues yielding glutamic acid residues during digestion of gliadin could result in an increased presentation of gliadin peptides by the DQ2 molecule. We studied *in vitro* the deamidation of glutamine residues in the synthetic A-gliadin peptide (45-56) using acidic conditions comparable to the gastric environment. Hydrolysis was analyzed by means of RP-HPLC, Edman degradation, capillary electrophoresis and electrospray ionization mass spectrometry. Furthermore, substitution analogs were synthesized carrying glutamic acid instead of glutamine. Finally, the binding to the DQ2 molecule was tested using the original gliadin peptide, peptides after hydrolysis and synthetic substituted peptides in a competition assay with purified HLA molecules[7].

Hydrolysis: A-gliadin peptide (45-56) (QPQPFPSQQPYL-NH_2) was synthesized on 4-methylbenzhydrylamine (MBHA)-resin using Fmoc chemistry, cleaved from the solid support by trifluoroacetic acid, containing 8 % of phenol, ethanedithiol and thioanisol (2:1:1) and precipitated by a cold mixture of *n*-heptane-diethylether (1:1)[9]. The crude peptide was analyzed by RP-HPLC and electrospray mass spectrometry (ESI-MS) and purified to 95 % homogeneity by means of RP-HPLC. Acidic treatment of the purified gliadin peptide was carried out at various conditions: 1 N HCl for 1.5 h or 3 h at 37 °C and 0.015 N HCl (corresponding to pH 1.8) for 3 h at 37 °C. Analysis by RP-HPLC gave a first indication of time-dependent chemical modifications in this peptide. Although the signals of the original gliadin peptide and of the resulting derivatives were strongly overlapping, ESI-MS also provided a qualitative evaluation of Q→E hydrolysis. Especially, hydrolysis in the peptide backbone leading to fragmentation during incubation could be clearly excluded.

Capillary electrophoresis: After acidic treatment, samples were analyzed by capillary electrophoresis (CE). Depending on the net charge of a peptide, this technique turned out to be the most suitable method for the quantification of Q→E hydrolysis, as deamidation of glutamine residues introduces an additional negative charge into the gliadin peptide. Prior to CE analysis, the peptide mixtures were desalted to remove sodium chloride resulting from neutralization of the incubation mixture with a 1 N NaOH solution. Quantitative evaluation of glutamine hydrolysis was based on the integrated peak area percentages. For gliadin peptide (45-56), a high rate of Q→E hydrolysis was found, even at pH 1.8 (35 % increase of deamidated peptides as compared to the untreated gliadin peptide). The relative amount of deamidated peptides was 45 % and 59 % after 1.5 h and 3 h incubation in 1 N HCl, respectively. The obtained electropherograms are shown in Figure 1.

Edman degradation: The direct confirmation of Q→E hydrolysis in the gliadin peptide (45-56) was achieved by N-terminal peptide sequencing and, furthermore, the extent of hydrolysis could be quantified at each glutamine position. The integrated peak heights were recorded for all PTH-amino acids at each sequence position and the ratio of the peak integrals for glutamine (Q) and glutamic acid (E) was calculated for each Q position (Q/E). Quantification of hydrolysis rates was performed by calculating the percent differences in Q/E values of the peptide mixture after acidic treatment and the Q/E values obtained by sequencing the peptide without incubation. Every sample was sequenced three times and the standard deviation of the calculated Q/E ratios was within 2.5 %. As determined with CE, sequencing data confirmed a high Q→E hydrolysis rate induced by acidic incubation and, in addition, the four glutamine residues showed different sensitivities to hydrolysis conditions: at pH 1.8, residue Q-47 was deamidated up to 11 %, Q-52 up to 6 %, while Q-45 and Q-53 were only weakly affected (2 %). After 1.5 h incubation in 1 N HCl, 20 % to 30 % Q→E hydrolysis was detected for all glutamine residues. However, the kinetic differences, representing different sensitivities of the glutamine residues to acidic hydrolysis, remained identical to those observed at pH 1.8 (0.015 N HCl). These differences decreased after prolonged incubation (3 h) in 1 N HCl.

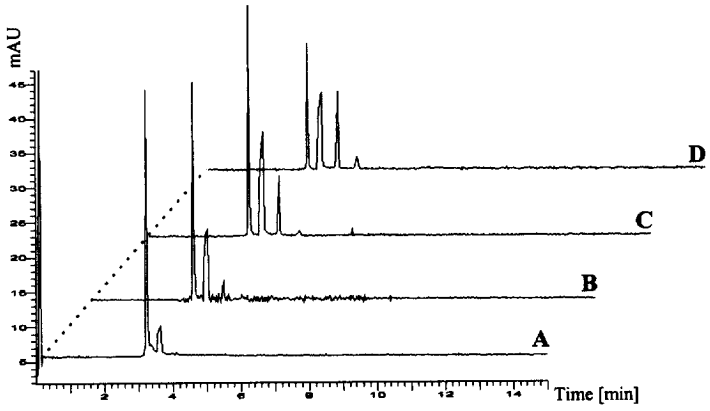

Figure 1. Capillary electrophoresis of gliadin peptide (45-56) after various incubation conditions (CE conditions: 20 mM phosphate buffer, pH 6.0, 10 kV, 214 nm UV detection): **A**, without incubation; **B**, incubation in HCl, pH 1.8, 3 h, 37 °C; **C**, incubation in 1 N HCl, 1.5 h, 37 °C; **D**, incubation in 1 N HCl, 3 h, 37 °C.

Peptide binding to HLA-DQ2: A competitive peptide binding assay was performed using europium fluoroimmunodetection[10]. In this assay, the measured fluorescence intensity corresponds to the DQ2-binding of the biotinylated indicator peptide, Bio-KPLLIIAEDVEGEY-NH_2 (MB 65 kDa 243-255Y peptide[5]). Thus, a high intensity in the presence of a competitor peptide (300-fold molar excess) indicates a poor affinity of the

competitor and vice versa. Measurements were performed in duplicates and standard deviation was within the limit of 7 %. DQ2-binding of the peptide mixture after acidic incubation and binding of synthetic Q→E substitution analogs were compared to DQ2-binding of the original gliadin peptide (45-56) (Fig. 2), resulting in a *relative competition*.

After incubation at pH 1.8 for 3 h, the hydrolysis mixture showed an increased binding of 28 % compared to gliadin peptide (45-56). Surprisingly, an increase of only 15 % was observed with the mixture obtained after 3 h incubation in 1 N HCl. For the synthetic analog of gliadin peptide (45-56) (Q-45→E-45), a *relative competition* of 20 % was recorded. The peptide analog with all glutamine residues replaced by glutamic acid showed an increased affinity of 41% to the DQ2 molecule.

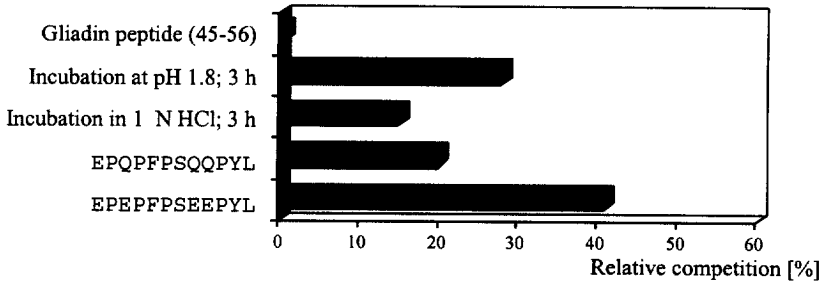

Figure 2. Competitive binding of the gliadin peptide and peptide pools to the CD-associated DQ2 molecule. *Relative competition* values express the increased binding of peptide pools and substitution analogs compared to the data obtained with the untreated gliadin peptide (45-56).

The influence of acid-induced chemical modifications in gliadin peptide (45-56) with respect to DQ2-binding was assessed using several analytical techniques and a cell-free DQ2 binding assay. This peptide was selected on the basis of its *in vitro* activity[11]. Moreover, its sequence comprises the tetrapeptide PSQQ, which is described, together with QQQP, as the longest sequence common to CD-inducing gliadin peptides[1,11]. Imitating an acidic environment like in the stomach, gliadin peptide (45-56) was submitted to hydrochloric acid treatment in various conditions. Thereby, a pool of peptidic products containing glutamic acid residues instead of glutamine residues was generated. According to the DQ2-binding motif displaying negatively charged residues at several anchor positions ligands with increased DQ2-affinity could be expected.

Analytical data confirmed Q→E hydrolysis even under mild conditions (such as gastric pH 1.8). From RP-HPLC and ESI-MS, only qualitative confirmation of hydrolysis events was obtained excluding backbone hydrolysis during incubation. However, CE and N-terminal peptide sequencing allowed quantification of the deamidation. An optimal separation of the glutamine-rich gliadin peptide (45-56) and deamidated products was achieved by CE (Fig. 1), clearly indicating Q→E hydrolysis. The hydrolysis rate of each of the individual

glutamine residues was determined by Edman degradation. Significant Q→E hydrolysis was observed for residues Q-47 (11 %) and Q-52 (6 %).

Preferential hydrolysis at defined positions might be related to the secondary structure of the gliadin peptide. Conformational studies on some natural and synthetic gliadin peptides demonstrated β-turns as the predominant structural feature and an involvement of β-turns in the induction of celiac disease was also suggested[12]. Considering the prediction of secondary structure in gliadin peptide (45-56) according to Chou and Fasman[13], β-turns are expected in the region F-49 to Q-52 (probability 2.18 x 10^{-4}) and Q-53 to L-56 (1.13 x 10^{-4}). Thus, under mild acidic conditions, the site of deamidation in synthetic gliadin peptides may be related to the particular conformation of these peptides.

DQ2-binding studies using the gliadin peptide (45-56), the peptide pools resulting from acid-treatment and synthetic Q→E substitution analogs as competitors confirmed the importance of Q→E hydrolysis for peptide-DQ2 interaction. The peptide pool obtained after 3 h incubation in 1 N HCl showed a 15 % higher competition compared to the original gliadin peptide (45-56). However, a stronger increase of 28 % in DQ2-binding was observed with the mixture obtained after 3 h incubation at pH 1.8. This confirms that DQ2 ligands can be generated by chemical alterations such as deamidation of glutamine residues under acidic conditions. A *relative competition* of 20 % was found for the synthetic peptide EPQPFPSQQPYL, that can only be explained by a positive contribution of glutamic acid in the anchor position 1 of DQ2 ligands. Although this is not consistent with the suggested DQ2-binding motif[7,8], these data are confirmed by our preliminary results in investigating the DQ2-peptide binding properties by means of synthetic peptide libraries[14]. The nonapeptide amide sublibrary with glutamic acid as the only defined residue at the N-terminus showed a three times higher competition than the sublibrary carrying a defined glutamine residue at this position. As expected, the peptide with four Q→E substitutions displayed a much higher DQ2-affinity than gliadin peptide (45-56). Residues E-52 and E-53 fit to DQ2 anchor positions 6 and 7, which preferentially interact with negatively charged residues[7,8]. Consequently, E-47 and Y-55 correspond to anchor positions 1 and 9, respectively. However, this peptide with all glutamine residues hydrolyzed to glutamic acid residues might be found in a very low concentration in the peptide pool obtained after acidic conditions at pH 1.8.

The data obtained with gliadin peptide (45-56) suggest that exposure to gastric acidity after ingestion of gliadin probably results in selective deamidation of glutamine residues. *In vivo* however, gliadin peptides are also exposed to gastric and pancreatic enzymes, resulting in further proteolytic degradation and thus, also peptides too short for efficient HLA class II binding might be generated. Glutamine deamidation can also occur after cellular uptake, when gliadin peptides pass intracellular acidic compartments involved in antigen processing. Furthermore, different enzymes in endosomes, lysosomes or in the MHC class II compartment might be directly involved in selective hydrolysis with regard to the peptide sequence and/or conformation of longer gliadin peptides. Thus, new T cell epitopes from gliadin peptides are generated and presented by the CD-

associated DQ2 molecule. Further work is in progress with other partially overlapping synthetic peptides from the N-terminal domain of A-gliadin and will also include studies on the intracellular processing of gliadin peptides.

Acknowledgements

The Swiss National Science Foundation is gratefully acknowledged for a postdoctoral research grant to C. T. The authors like to thank Ms. A. Torun and Ms. M. Wurster for assistance in peptide synthesis and CE analysis, respectively. We thank Prof. M. Stern and D. Stoll for suggestions and critical reading of the manuscript.

References

1. Marsh, M. N. *Gastroenterology* **1992**, *102*, 330.
2. Sollid, L. M.; Markussen, G.; Ek, J.; Gjerde, H.; Vartdal, F.; Thorsby, E. *J. Exp. Med.* **1989**, *169*, 345.
3. Sollid, L. M.; Thorsby, E. *Gastroenterology* **1993**, *105*, 136.
4. Lundin, K. E. A.; Scott, H.; Hansen, T.; Paulsen, G.; Halstensen, T. S.; Fausa, O.; Thorsby, E.; Sollid, L. M. *J. Exp. Med.* **1993**, *178*, 187.
5. Johansen, B. H.; Gjertsen, H. A.; Vartdal, F.; Buus, S.; Thorsby, E.; Lundin, K. E. A.; Sollid, L. M. *Clin. Immunol. Immunopathol.* **1996**, *79*, 288.
6. Shidrawi, R. G.; Parnell, N. D. J.; Ciclitira, P. J.; Travers, P.; Evan, G.; Rosen-Bronson, S. *Clin. Exp. Immunol.* **1998**, *111*, 158.
7. van de Wal, Y.; Kooy, Y. M. C.; Drijfhout, J. W.; Amons, R.; Koning, F. *Immunogenetics* **1996**, *44*, 246.
8. Vartdal, F.; Johansen, B. H.; Friede, T.; Thorpe, C. J.; Stevanovic, S.; Eriksen, J. A.; Sletten, K.; Rammensee, H.-G.; Sollid, L. M. *Eur. J. Immunol.* **1996**, *26*, 2764.
9. Jung, G.; Beck-Sickinger, A. G. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 367.
10. Tompkins, S. M.; Rota, P. A.; Moore, J. C.; Jensen, P. E. *J. Immunol. Meth.* **1993**, *163*, 209.
11. Wieser, H. *Acta Paediatr.* **1996**, *Suppl. 412*, 3.
12. Tatham, A. S.; Marsh, M. N.; Wieser, H.; Shewry, P. R. *Biochem. J.* **1990**, *270*, 313.
13. Chou, P. Y.; Fasman, G. D. *Annu. Rev. Biochem.* **1978**, *47*, 251.
14. Fleckenstein, B.; Kalbacher, H.; Muller, C. P.; Stoll, D.; Halder, T.; Jung, G.; Wiesmüller, K.-H. *Eur. J. Biochem.* **1996**, *240*, 71.