Journal of Chromatography A, 1391 (2015) 60–71

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Label free targeted detection and quantification of celiac disease immunogenic epitopes by mass spectrometry

Hetty C. van den Broeck[a,*], Jan H.G. Cordewener[a], Merel A. Nessen[b], Antoine H.P. America[a], Ingrid M. van der Meer[a]

[a] Wageningen UR, Plant Research International, PO Box 16, 6700 AA Wageningen, The Netherlands
[b] RIKILT Wageningen UR, PO Box 230, 6700 AE Wageningen, The Netherlands

ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form 23 February 2015
Accepted 26 February 2015
Available online 5 March 2015

Keywords:
α-Gliadin
Celiac disease
LC-MRM/MS
T-cell stimulatory epitopes
Wheat

ABSTRACT

Celiac disease (CD) is a food-related disease caused by certain gluten peptides containing T-cell stimulating epitopes from wheat, rye, and barley. CD-patients have to maintain a gluten-free diet and are therefore dependent on reliable testing and labeling of gluten-free products. So far, the R5-ELISA is the approved method to detect if food products can be labeled gluten-free. Because the R5-ELISA detects gluten in general, there is a demand for an improved detection method that quantifies specifically CD-epitopes. Therefore, we developed a new method for detection and quantification of CD-epitopes, based on liquid chromatography (LC) coupled to mass spectrometry (MS) in multiple reaction monitoring (MRM) mode. This method enables targeted label free comparative analysis of the gluten proteins present in different wheat varieties and species, and in wheat-based food products. We have tested our method by analyzing several wheat varieties that vary in CD-epitope content, as was shown before using immunoblotting and specific monoclonal antibodies. The results showed that a modern bread wheat variety Toronto contained the highest amounts of CD immunogenic peptides compared with the older bread wheat variety Minaret and the tetraploid wheat variety Dibillik Sinde. Our developed method can detect quantitatively and simultaneously multiple specific CD-epitopes in a high throughput manner.

1. Introduction

Celiac disease (CD) is a food-related disease that results in inflammation of the small intestinal mucosa in genetically predisposed individuals caused by intake of gluten proteins from wheat, rye, and barley [1]. The prevalence of celiac disease is about 0.5–2% and is still increasing in Western parts of the world as well as in developing countries [2,3]. CD-patients can present symptoms such as diarrhea, abdominal pain, constipation, weight loss, and dermatitis herpetiformis, however, increasingly more patients show asymptomatic presentation without gastrointestinal symptoms resulting in a high number of undiagnosed cases [4–7]. For CD-patients still the only cure is to adhere to a strict gluten-free diet. However, gluten proteins are increasingly being applied in all kind of food products because of their interesting features [8]. Therefore, it is very important that food labeling is accurate and reliable. The reduced intake of gluten peptides and proteins containing CD-epitopes by all consumers, including diagnosed and still undiagnosed CD-patients, will reduce symptoms and may reduce the prevalence of CD.

Gluten proteins, i.e. gliadins and glutenins, represent the main part of storage proteins in wheat, are insoluble in water and contain high percentages of the amino acids proline (P) and glutamine (Q). Gliadins form a large protein family in which α/β-, γ-, and ω-gliadins can be distinguished (~30 to ~80 kDa by acidic-PAGE) [9,10]. Glutenins can be subdivided into low-molecular weight glutenin subunits (LMW-GS; ~30 to ~70 kDa by SDS-PAGE) and high-molecular weight glutenin subunits (HMW-GS; ~80 to ~130 kDa by SDS-PAGE) [11,12].

Bread wheat is hexaploid and contains three different genomes (A, B and D) evolved from three different grass species. Therefore, many gluten protein encoding genes are present and copy numbers for α-gliadins can range between 100 and 150 [13,14]. CD symptoms can be caused by many different CD-epitopes present in wheat cultivars and wheat-derived food products. So far, 24 epitopes have been defined from wheat gluten proteins that give a T-cell response in CD-patients [15]. These glutamine and proline rich epitopes are derived from all different classes of gliadin and glutenin proteins. The immunogenic epitopes present in α-gliadins are Glia-α2, Glia-α9, and Glia-α20 of which the most immunogenic epitopes are

* Corresponding author. Tel.: +31 317 480974; fax: +31 317 41809.
E-mail address: hetty.busink@wur.nl (H.C. van den Broeck).

http://dx.doi.org/10.1016/j.chroma.2015.02.070
0021-9673/

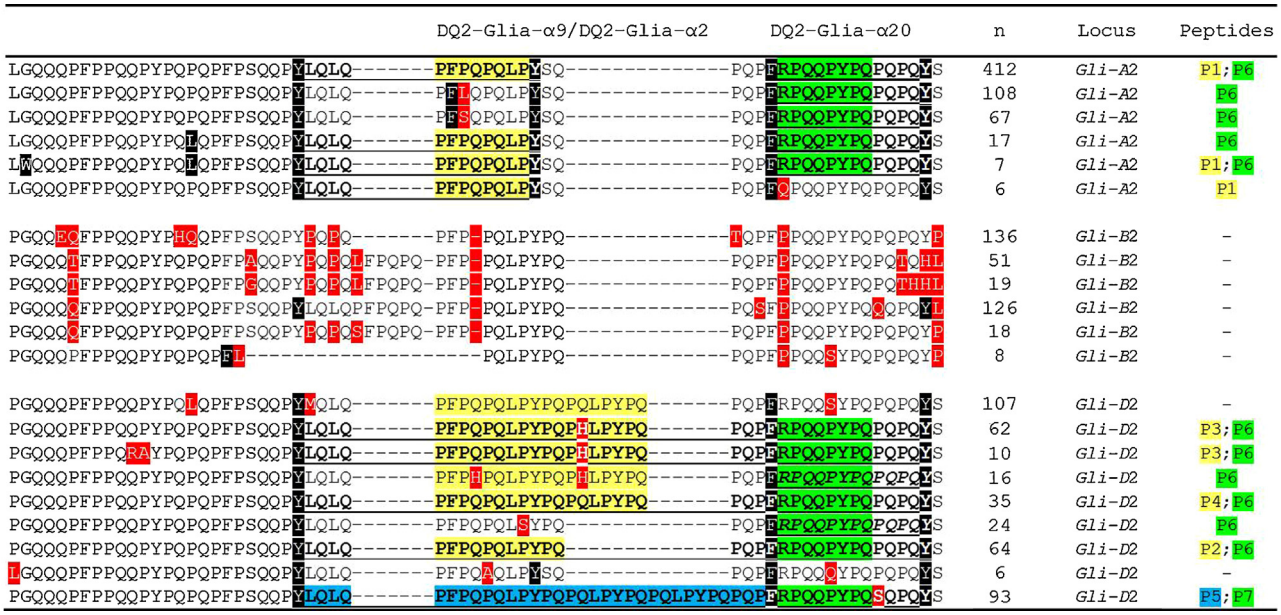

Fig. 1. Sequence alignment of the CD-epitope domain of multiple α-gliadin proteins from wheat. The deduced amino acid sequences of multiple EST contigs (present in the NCBI-UniGene database) were searched for sequence variants in the CD-epitope containing domain. Homologous ESTs for this sequence domain were grouped and only variants found in more than 5 ESTs (*n*) are displayed. The variants are grouped per genome origin from which the ESTs are expressed: Gli-A2, Gli-B2, or Gli-D2. The CD-epitopes, known to trigger the adaptive HLA-DQ2^{+} T-cell response (Glia-α2/Glia-α9, and Glia-α20) in CD-patients, are color indicated. In yellow: Glia-α2/Glia-α9 epitopes. In green: Glia-α20 epitope, in blue: 33-mer containing repetitive epitopes. In black: chymotrypsin digestion sites. In red: amino acid (AA) variation in the sequence. In bold underlined: sequences selected for peptide synthesis. The peptide number code is indicated in the right-most column.

Source: Figure is adapted from Mitea et al. [20].

Glia-α2 and Glia-α9 [16,17]. It has been shown by Shan et al. [18] that a large 33-mer from wheat gluten is resistant against human intestinal proteases and therefore can be present in the small intestine. The 33-mer is composed of overlapping immunodominant Glia-α2 and Glia-α9 epitopes that can also be recognized separately and stimulate T-cells in CD [16,17]. This 33-mer can be present in α-gliadins that are encoded by the *Gli*-2 locus (*Gli-D2*) on the short arms of chromosome 6 of the D-genome in wheat (see Fig. 1) [19–21]. The same holds for the Glia-α2 epitope that is present in most α-gliadins encoded by genes present at only the D-genome in hexaploid wheat. The α-gliadins encoded by the D-genome can also contain derivatives of the 33-mer (a 19-mer and a 26-mer) that contain the Glia-α2 and Glia-α9 epitopes. The α-gliadins encoded by the A-genome in hexaploid and tetraploid wheat contain only a 13-mer containing the Glia-α9 epitope. Most α-gliadins encoded by both the A- and the D-genome contain the Glia-α20 epitope. The α-gliadins encoded by the B-genome contain neither the Glia-α2/9 epitopes nor the Glia-α20 epitope [20,22].

Food products can be labeled gluten-free if they contain less than 20 ppm gluten. So far, the only approved test by the Codex Alimentarius to detect the presence of gluten in food products and that can be used to label products 'gluten-free' is the R5-enzyme-linked immunosorbent assay (R5-ELISA) [23–25]. In general, the functionality of ELISA tests depends on the extraction protocol [26–28], reference material for calibration [29], and the detecting antibody. The latter is one of the limitations of the R5-ELISA that makes use of a monoclonal antibody that detects gluten in general and not specifically CD stimulatory epitopes. The R5-antibody is a-specific and recognizes several, five amino acids long, sequences present in gluten proteins from wheat, rye, and barley, but not from soy, oats, corn, rice, millet, teff, buckwheat, quinoa, and amaranth. This difference in specificity is essential for gluten-free testing. Despite this ability, the short length of the recognition sequence might lead to inaccurate results. Antibodies can recognize sequences as short as five amino acids. This in contrast to the size of the recognition sequence of a CD-epitope recognized by human T-cells in CD, which is at least nine amino acids in length [30]. Therefore, R5-ELISA might result in an overestimation of the amount of true T-cell epitopes present that stimulate CD.

Simultaneous detection and quantification of more than one CD-epitope is not possible by immunoblotting using a single blot. By ELISA this is only possible when a multiplexing method with multiple specific antibodies is used. Therefore, the development of a liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM/MS) method that can detect and quantify multiple CD-peptides in a single short run will have a huge advantage over the existing methods.

LC-MRM/MS is a rapidly emerging method as an alternative to antibody based protein quantification [31–33]. In this approach, the proteins to be quantified are first digested with a specific protease, after which proteotypic peptides are analyzed by MS in MRM mode. Using either a triple quadrupole or a quadrupole-ion trap instrument, peptides are identified and quantified by monitoring several transitions for each peptide. MRM allows sensitive, accurate and reproducible quantification of the peptides and corresponding proteins.

So far, several LC–MS detection methods, aiming at the detection of different immunogenic peptides, have been developed and described [34–39]. The developed methods differ in the choice of the selected peptides for quantification and the protease treatments to release the peptides from the gluten proteins.

Here, we describe the development of an LC-MRM/MS method to quantify individual immunogenic peptides in a gluten protein extract from wheat kernels, using chymotrypsin digestion to release the peptides. We focused on detection of those peptides that have been proven to be the most immunodominant in CD [40–44]. This is in contrast to previously developed LC–MS methods, that focused on the most intensely MS responding peptides. Besides immunodominant peptides, we also incorporated some peptides with amino acid substitutions that make the immunogenic epitopes inactive and are thereby considered safe for CD-patients [20,22]. LC–MS allows detection of these amino acid substitutions, which