Food Chemistry 214 (2017) 597–605

Contents lists available at ScienceDirect

Food Chemistry

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

Oxidation of proline decreases immunoreactivity and alters structure of barley prolamin

Xin Huang [a,*], Tuula Sontag-Strohm [a], Frederick L. Stoddard [a], Yoji Kato [b]

[a] Department of Food and Environmental Sciences, University of Helsinki, Agnes Sjöberginkatu 2, PL 66, Helsinki FI-00014, Finland
[b] School of Human Science and Environment, Research Institute for Food and Nutritional Sciences, University of Hyogo, Himeji, Hyogo 670-0092, Japan

ARTICLE INFO

Article history:
Received 10 May 2016
Received in revised form 14 July 2016
Accepted 19 July 2016
Available online 19 July 2016

Keywords:
Barley
Hordein
Proline oxidation
Proline
Gluten-free
Metal-catalyzed oxidation
Prolamin

ABSTRACT

Elimination of celiac-toxic prolamin peptides and proteins is essential for Triticeae products to be gluten-free. Instead of enzymatic hydrolysis, in this study we investigated metal-catalyzed oxidation of two model peptides, QQPFP, and PQPQLPY, together with a hordein isolate from barley (*Hordeum vulgare* L.). We established a multiple reaction monitoring (MRM) LC–MS method to detect and quantify proline oxidation fragments. In addition to fragmentation, aggregation and side chain modifications were identified, including free thiol loss, carbonyl formation, and dityrosine formation. The immunoreactivity of the oxidized hordein isolate was considerably decreased in all metal-catalyzed oxidation systems. Cleavage of peptides or protein fragments at the numerous proline residues partially accounts for the decrease. Metal-catalyzed oxidation can thus be used in the modification and elimination of celiac-toxic peptides and proteins.

1. Introduction

Degradation of cereal prolamin proteins and peptides can reduce their toxicities for celiac disease (CD) patients. Barley prolamins, called hordeins, are toxic for CD patients. Hordein consists of the polymers, B-hordein and D-hordein and the monomers, C-hordein and γ-hordein, with B-hordein and C-hordein together comprising over 80% of the total hordein. Proteolytic enzymes, such as proteases from seed germination (Loponen et al., 2009) and proline-endopeptidase (Luoto et al., 2012) may be used for prolamin degradation. We have previously demonstrated that metal-catalyzed oxidation degraded a model celiac peptide 33-mer (Huang, Kanerva, Salovaara, Loponen, & Sontag-Strohm, 2013) and C-hordein (Huang, Kanerva, Salovaara, & Sontag-Strohm, 2016), as shown by substantial reductions in the immunoreactivity of both substrates in R5-based enzyme-linked immunosorbent assay (ELISA). Protein degradation and aggregation both occurred during oxidation, and oxidation of proline residues could partially explain this phenomenon.

For this study of metal-catalyzed oxidation, we selected two model peptides, QQPFP and PQPQLPY, along with barley prolamin (hordein). The sequence QQPFP is the repetitive domain in omega-type prolamins in wheat, barley and rye, including ω-gliadin, C-hordein, and ω-secalin (Tatham & Shewry, 1995). Gamma-type and alpha-type prolamins also contain the sequence QQPFP, but at a lower frequency (Osman et al., 2001). The current official method for quantification of prolamins in food is based on an antibody against this epitope, QQPFP (Codex Alimentarius Commission 118-1979). In peptides that are known to be harmful for CD patients, QQPFP is often found in the sequence, along with other similar sequences including QQQP, QQPYP, PQQPY, and QQQPFP (Cornell & Stelmasiak, 2007). These sequences are involved in the triggering of T-cell activation after deamidation in both *in vitro* and *in vivo* studies (Arentz-Hansen et al., 2002). The other model peptide in this work, PQPQLPY, is the repetitive sequence in the 33-mer peptide that has been used as a model in celiac studies (Shan et al., 2002), and the recognition sequence for the G12 antibody that is the basis for the quantification method for gluten recently approved by AACCI (method 38-52.01). This peptide overlaps the T-cell stimulating epitopes PFPQPQLPY, PYPQPQLPY, and PQPQLPYPQ (Shan et al., 2002). The presence of glutamine, proline, and either of the aromatic amino acids, phenylalanine or tyrosine, makes an important site for recognition of either the R5 or the G12 antibody, or activation of T-cells (Morón et al., 2008). The gluten content of the barley products, such as malt, malt extract or beer, has to be under 20 mg/kg to be labelled as gluten-free. Each hordein protein and subunit contains epitopes that are toxic for CD patients (Vader et al., 2003).

* Corresponding author.
E-mail address: xin.huang@helsinki.fi (X. Huang).

http://dx.doi.org/10.1016/j.foodchem.2016.07.108
0308-8146/

The aim of this study was to investigate the modification of celiac peptides and proteins by metal-catalyzed oxidation, in order to reduce their immunoreactivities. We examined the contribution of proline fragmentation to the oxidation of model peptides and whole hordein, the side-chain modifications and aggregation behavior during oxidation, and the immunoreactivity of the oxidation products against R5 antibody.

2. Materials and methods

2.1. Materials

The synthetic peptides QQPFP (MW = 615.68 g/mol, purity 97.2%), and PQPQLPY (MW = 841.95 g/mol, purity 98.6%) were purchased from GenScript (Piscataway, NJ, USA). Gamma aminobutyric acid (GABA), $FeSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), diethylene triamine pentaacetic acid (DTPA), 2,4-dinitrophenylhydrazine (DNPH), and dithiothreitol (DTT) were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Preparation of hordein isolate

Barley cv. Jorma was obtained from Villala, Finland and grains were milled with a sample mill (Koneteollisuus Oy, KT-30, Finland). Hordeins were extracted from the flour following the Osborne sequence of milli-Q water, 0.5 mol/l NaCl, and milli-Q water at ambient temperature and then 60% ethanol with 0.1% DTT at 50 °C. After centrifugation for 10 min at 18,500×*g*, the supernatant was collected and dialyzed against milli-Q water with membrane cut-off MW = 3500 for 48 h at 4 °C, and the water was changed at least three times. The dialyzed protein was lyophilized (Heto-Holton DW8-85, Denmark). The protein solids were the starting material for oxidation.

2.3. Oxidation of hordein and model peptides

Hordein at 2 mg/ml, and the synthetic peptides QQPFP and PQPQLPY at 2.5 mmol/l were separately incubated in milli-Q water at 40 °C in the presence of either 0.05 mmol/l $CuSO_4$ or 0.05 mmol/l $FeSO_4$/EDTA (1:1). The reaction was initiated by either 5 mmol/l freshly made hydrogen peroxide (H_2O_2) or 5 mmol/l ascorbic acid (AA). After incubation, the reaction was terminated by adding 0.1 mmol/l EDTA for $CuSO_4$-catalyzed oxidation, and 0.1 mmol/l DTPA for $FeSO_4$/EDTA-catalyzed oxidation. There were thus four treatments, Cu/AA, Cu/H_2O_2, $FeSO_4$/EDTA/AA, and $FeSO_4$/EDTA/H_2O_2.

2.4. Identification of proline fragments by LC–MS

The peptide oxidation mixtures were analyzed using an LC–MS/MS system. An Agilent 1100 HPLC system coupled with a Develosil ODS-SR-5 column (4.6 × 150 mm, Nomura Chemical, Co., Ltd, Japan) was connected with a triple quadrupole mass spectrometer (API 3000, AB Sciex), in positive mode. An acetonitrile gradient from 100% solvent A (0.1% formic acid in water) to 50% solvent B (0.1% formic acid in acetonitrile) was run over 20 min at a flow rate of 0.2 ml/min. The structures of QQPFP and the proposed products F1 and F2 are illustrated in Fig. 1. The multiple reaction monitoring (MRM) method setting for QQPFP was Q1 *m*/*z* 616.3 → Q3-I *m*/*z* 360.5, and Q3-II *m*/*z* 376.5; for product F1 was Q1 *m*/*z* 342.2 → Q3-I *m*/*z* 197.2, and Q3-II *m*/*z* 86.2; for product F2 was Q1 *m*/*z* 586.3 → Q3 *m*/*z* 330.3. Two products of PQPQLPY were proposed, product F3 from attack at the middle proline, and product F4 from attack near the C-terminal proline (structures not shown). The MRM setting for PQPQLPY was Q1 *m*/*z* 842.4 → Q3-I *m*/*z* 564.3 and Q3-II *m*/*z* 226.1, for F3 was Q1 *m*/*z* 311.2 → Q3-I *m*/*z* 196.4 and Q3-II *m*/*z* 86.2, and for F4 was Q1 *m*/*z* 649.4 → Q3-I *m*/*z* 424.4 and Q3-II *m*/*z* 226.4. The MRM settings were obtained from direct infusion of the oxidized mixture to the mass spectrometer.

The product of proline fragmentation is presumed to be 2-pyrrolidone, but MRM alone does not identify it. After acid hydrolysis, 2-pyrrolidone opens to GABA (Kato, Uchida, & Kawakishi, 1992a, 1992b), enabling identification of the proline fragment.

In order to identify the proline fragment products, the reaction mixture was separated in a semi-preparative C18 column (8.0 × 250 mm, Develosil ODS-HG-5, Nomura). The gradient was solvent B (A and B as above) from 0% to 50% over 34 min at a flow rate of 2 ml/min. The peaks containing fragment products were collected and concentrated in a centrifugal evaporator (EYELA, UNITRAP-UT-2000, TOMY centrifugal concentrator CC-105). The dried fragments were acid-hydrolyzed by 6 mol/l HCl under vacuum at 105 °C for 24 h, in the presence of 1% phenol as antioxidant and 5% mercaptoacetic acid. After acid hydrolysis, GABA was identified by the MRM method. The MRM setting for GABA was Q1 *m*/*z* 103.8 → Q3-I *m*/*z* 87.0, and Q1 *m*/*z* 103.8 → Q3-II *m*/*z* 69.0 (Buck, Voehringer, & Ferger, 2009).

The GABA formation from hordein oxidation was then quantified. The reaction mixture was precipitated with 5 times volume of cold acetone for 10 min. After centrifugation at 22,000×*g*, the pellets were acid-hydrolyzed as described above and the product was separated on an Amide 80 column (TSKgel, 3 μm, 2.0 × 150 mm, TOSOH LLC), on a gradient of 15% solvent A (2 mmol/l ammonium formate in milli-Q water) to 50% solvent B (acetonitrile) over 20 min at a flow rate of 0.15 ml/min. As an internal standard, 250 nmol/l $^{13}C_9$ L-tyrosine was added to all the standards and samples. The amount of GABA was determined with an amino acid analysis kit (EZFaast, Phenomenex, Torrance, USA) with LC–MS/MS following the manufacturer's instructions. The hydrolysates were performed in triplicate and each replicate was analyzed twice. Total GABA and valine residues were also calculated. Changes in GABA content were calculated using valine content as a reference.

2.5. Size-exclusion chromatography of hordein oxidation

The size distribution of the oxidized mixture was analyzed with two connected columns, Superdex peptide 10/300 GL and Superdex 200 10/300 GL (GE Healthcare Biosciences, Uppsala, Sweden) as described previously (Huang et al., 2016).

2.6. Blot analysis of free thiol groups

The hordein oxidation mixture was treated with a final concentration of 200 μmol/l EZ-link Iodoacetyl-LC-Biotin (PIERCE 21333, Rockford, IL, USA) for 30 min at ambient temperature avoiding light (King et al., 1992). After incubation, the protein was precipitated with cold acetone at 10 times the volume of reaction mixture and centrifuged at 22,000×*g*. The protein pellet was washed by resuspending in cold acetone and recentrifuging to remove the extra biotin. The pellet was dissolved in SDS-Sample buffer (×2) with reducing agent 5% (v/v) 2-mercaptoethanol. The protein sample was separated on a 10% polyacrylamide gel at 200 V for 40 min. The gel was blotted to a polyvinylidene difluoride membrane in a semi-dry system (TransferBlot SD, Bio-Rad) at a constant 150 mA for 40 min. After the membrane was blocked with 1% (m/v) gelatin solution for 1 h, it was incubated for 30 min in streptavidin-peroxidase polymer (ultrasensitive, SIGMA S2438) diluted