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Production of gluten-free wheat starch by peptidase treatment

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ABSTRACT

The potential of peptidase-containing bran extracts from germinated cereals (wheat, emmer, barley) and a peptidase preparation from *Aspergillus niger* (AN-PEP) to degrade gluten in wheat starch below the threshold for gluten-free foods of 20 mg/kg was compared. The gluten-specific peptidase activity of the peptidases was determined by using gliadin as a protein-based substrate as well as the two celiac-active peptides PQPQLPYPQPQLPY (α-gliadin) and SQQQFPQPQQPFPQQP (γ-hordein). The peptidase activity of AN-PEP exceeded the activities of bran from germinated cereals by a factor up to 690,000. Three wheat starches with initial gluten contents of 110, 1679, and 2070 mg/kg, respectively, were incubated with bran extracts and AN-PEP, lyophilized, and residual gluten was quantitated by a competitive ELISA. Unlike peptidases from bran extracts, AN-PEP was capable of degrading gluten below 20 mg/kg in all starches. The absence of gluten in AN-PEP-treated starches was confirmed by liquid-chromatography-mass spectrometry. The properties of gluten-free starches were comparable to the native starches with the exception of a reduced viscosity after AN-PEP treatment. This problem could be overcome by using higher enzyme concentrations and shorter incubation times or by optimizing AN-PEP production for lower residual α-amylase activity.

1. Introduction

Celiac disease (CD) is a life-long immune-mediated enteropathy occurring in genetically predisposed individuals. It is triggered by the ingestion of storage proteins, called gluten in the field of CD, present in wheat, rye, barley, and oats (for recent review see Wieser et al., 2012). Originally thought to occur only rarely in children, CD is now recognized as a common condition at any age. The estimated prevalence of CD in the Western population is approximately 1%, and, thus, CD is one of the most frequent food intolerances. Permanent adherence to a gluten-free diet is the only and currently essential treatment of CD. As the amount of gluten tolerated by CD patients varies considerably, there is no need to suggest a threshold for a single dose. A daily intake of less than 10 mg gluten, however, is unlikely to cause significant symptoms (Akobeng and Thomas, 2008). The diet is difficult to sustain due to restricted availability, higher costs, and poor quality of gluten-free food alternatives regarding taste, flavor, and mouth feel.

The Codex Alimentarius as well as European legislation (Codex Alimentarius Commission, 2008; European Commission, 2009) have set the threshold for a "gluten-free" claim at 20 mg gluten/kg product. Products with up to 100 mg/kg can be declared as "low in gluten". However, despite this strict regulation, even those CD patients that are accurately adherent to a gluten-free diet, might consume more than the tolerable amount of gluten as a result of contamination and "hidden" gluten (e.g., in thickened sauces and soups, puddings or sausages). Therefore, it is desirable to provide gluten-free products with a gluten level as low as possible. Recent research has shown that different proline-specific endopeptidases can be used for gluten degradation, not only in oral therapy of CD but also for the elimination of gluten in raw materials and foods (reviewed by Wieser and Koehler, 2012). Germinated cereal grains as well as bacteria and fungi are known to be suitable sources for proline-specific peptidases (Shan et al., 2002; Hartmann et al., 2006; Edens et al., 2005a).

Wheat starch is one of the most frequently used ingredients of gluten-free baked goods and appreciated because of its favorable technological properties. In comparison to starches from gluten-free sources such as rice, corn or potatoes, wheat starch provides higher water absorption, dough density, and bread volume

Abbreviations: AN-PEP, Aspergillus niger prolyl endopeptidase; CD, celiac disease; DSC, Differential Scanning Calorimetry; DTE, dithioerythritol; LC-MS, liquid chromatography - mass spectrometry; P1, peptide PQPQLPYPQPQLPY; P2, peptide SQQQFPQPQQPFPQQP; P3, peptide VQGQGIIQPQQPAQL; P4, peptide VQQGIIQPQQPAQL; RP-HPLC, reversed-phase high-performance liquid chromatography; RT, room temperature; RVA, Rapid Visco Analyzer; SIM, selected ion monitoring mode; TFA, trifluoroacetic acid; U, Unit (enzyme activity).

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(Houben et al., 2012). However, wheat starch may contain residual gluten and its level depends on the production process (e.g. number of washing steps) and can vary in a wide range from below 20 to more than 500 mg/kg (Wieser and Seilmeier, 2003) so that pre-treatment is necessary, before it can be used in the production of gluten-free foods. In addition, products such as starch syrup, maltodextrins, and glucose are produced from wheat starch on a large scale.

Therefore, the aim of the present study was to render wheat starch gluten-free by means of gluten-specific peptidases from bran extracts of germinated cereals and AN-PEP without altering its technological properties.

2. Material and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (Steinheim, Germany) or VWR (Darmstadt, Germany) at analytical or higher grade.

2.2. Starches

A commercially available wheat starch (starch 1) as well as starches from common wheat cv. Tommi (starch 2) and cv. Flair (starch 3) were used. The latter were obtained by washing dough (17 g) with distilled water (530 mL, 10 min) in a Glutomatic Type 2200 (Perten, Hägersten, Sweden) according to ICC standard 137/1 (1994). Suspended starch in the washing liquid was centrifuged (room temperature (RT) ≈ 20 °C, 20 min, 3760 *g*). After decanting the supernatant, the sediment was lyophilized and subsequently used as starch sample for enzymatic gluten degradation.

2.3. Enzymes

Enzymes from bran of germinated emmer cv. Osiris (germination for 7 days at 15 °C), barley cv. Marthe (germination for 7 days at 25 °C), and common wheat cv. Winnetou (germination for 7 days at 25 °C) were extracted from lyophilized cereal bran to give concentrations of 50–100 mg bran/mL. For this purpose, bran (250–500 mg) was extracted with sodium acetate buffer (5.0 mL; 0.2 mol/L, adjusted to pH 4.0 with 32% (w/w) hydrochloric acid) at 4 °C by means of vortexing (1 min) and subsequent magnetic stirring (15 min) according to Gessendorfer et al. (2011) and Schwalb et al. (2012). The suspension was centrifuged (RT, 20 min, 3760 *g*), the supernatant containing the peptidases was decanted, and filtered through a 0.45 μm membrane ("enzyme extract"). A commercially available proline-specific peptidase preparation from *Aspergillus niger* (Aspergillus Niger-Prolyl Endopeptidase, "AN-PEP"; DSM Food Specialties B.V., Delft, The Netherlands) was used for the degradation of gluten. The lyophilized fermentation broth of AN-PEP was dissolved in distilled water to obtain different concentrations (0.001–500 mg/mL).

2.4. Peptides

The peptides PQPQLPYPQPQLPY ("P1", M = 1665.86 g/mol) from α-gliadins (Arentz-Hansen et al., 2000) and SQQQFPQPQQPFPQQP ("P2", M = 1909.92 g/mol) from γ-hordeins (Vader et al., 2003) were used as substrates for determination of the gluten-specific peptidase activity. For the determination of residual gluten in treated wheat starches, VQGQGIIQPQQPAQL ("P3", M = 1604.87 g/mol) from γ-gliadins (Vader et al., 2002) and VQQGIIQPQQPAQL ("P4", M = 1547.85 g/mol) as an internal standard were used. All peptides were purchased from GenScript Corporation (Piscataway, NJ, USA). The purity given by the manufacturer was 95%.

2.5. Gluten-specific peptidase activity of enzyme extracts

The gluten-specific peptidase activity was determined according to Schwalb et al. (2012). P1 and P2 as well as the gliadin fraction from flour of common wheat cv. Cubus 2009 prepared according to Gessendorfer et al. (2009) were used as substrates. Briefly, gliadin (50 mg) was dissolved in 60% (v/v) ethanol (20 mL), incubated for 2.5 h at 50 °C, and filtered through a 0.45 mm membrane. Gliadin solution (130 μL) and peptidase solution (380 μL) were mixed including addition of a buffer to yield a final pH of 4.0 or 6.5 and magnetically stirred for 150 min at 50 °C. Incubated samples were filtered through a 0.45 μm membrane and analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a C_{18} silica gel column (2.1 × 150 mm, 3 μm, 30 nm; Dionex, Idstein, Germany) at 60 °C. The absorbance area of γ-gliadins at 210 nm was monitored and used as a measure for total gliadin as described by Schwalb et al. (2012). P1 or P2 (1.0 mg) was dissolved in buffer (5.0 mL; sodium acetate 0.2 mol/L, pH 4.0 or sodium dihydrogen phosphate 0.2 mol/L, pH 8.0 with 1 mol/L NaOH). Peptide solution (90 μL) and peptidase solution (60 μL) were mixed to yield a final pH of 4.0 or 6.5 and magnetically stirred for 60 min at 50 °C. Incubated samples were filtered through a 0.45 μm membrane and analyzed by RP-HPLC using a Nucleosil C_{18} column (3 × 250 mm, 5 μm, 10 nm, RT; Macherey–Nagel, Dueren, Germany) at 60 °C. The peptidase activity was determined on the basis of the decrease of the HPLC peak area of the peptides at 210 nm after incubation (Schwalb et al., 2012). The peptidase activity of AN-PEP towards all substrates was determined at a lower enzyme-to-substrate ratio. All determinations were made in duplicate. Enzyme activities used for calculation and discussion later in this article are based on gliadin as a substrate.

2.6. Gluten degradation in wheat starch

Wheat starch sample (5 g) was weighed in Pyrex glasses (30 mL), enzyme extract (50 μL) and distilled water (20 mL) was added, the pH value (range 1.0–9.0, in increments of 1.0) was adjusted with buffer (1 mL), the samples were left in a water bath at a defined temperature (4–60 °C) for 4–24 h under continuous agitation, and were lyophilized. The gluten content was analyzed before and after treatment at different temperatures (4–60 °C at pH 4.0) and pH values (1.0–9.0 at 50 °C). Buffers were as follows: pH 1, glycine-HCl-buffer, 0.2 mol/L; pH 2–4, sodium acetate-buffers, 0.2 mol/L; pH 5–8, phosphate-buffers, 0.2 mol/L; pH 9.0, Tris/HCl-buffer, 0.2 mol/L). The amount of enzyme was varied by increasing the concentration or the volume (up to 250 μL) of the enzyme solution (bran extracts 50–100 mg/mL, AN-PEP 0.001–500 mg/mL).

2.7. Gluten quantitation by ELISA

Gluten was quantitated by means of a competitive ELISA using the R5 antibody (RIDASCREEN® Gliadin competitive, R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions (R-Biopharm, 2007). Gliadin concentrations were calculated based on a cubic spline calibration function provided by the RIDA®SOFT Win software (R-Biopharm, Darmstadt, Germany) and converted into gluten concentrations by multiplying by a factor of two (Codex Alimentarius Commission, 2008).