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Degradation of Gluten in Wheat Bran and Bread Drink by Means of a Proline-Specific Peptidase

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Abstract

Gluten-free wheat bran and bread drink were produced by degrading gluten with *Aspergillus niger* prolyl endopeptidase (AN-PEP). For this purpose, bran from native and germinated wheat grains as well as bread drink were mixed with AN-PEP and incubated at 50°C under different conditions. The amount of enzyme activity ($1.2 \cdot 10^{-4}$ - $8.7 \cdot 10^{-1}$ U), the incubation time (0-72 h), and the pH value (1.0-9.0) were systematically altered. The gluten content was monitored by a competitive ELISA using the R5 antibody. Gluten in two wheat bran samples produced in the laboratory was degraded below the threshold for gluten-free foods of 20 mg/kg. This was not possible in a commercial wheat bran sample that had potentially been heat-treated leading to strong crosslinking of gluten by incorporation of gliadins into the glutenin fraction, possible formation of isopeptide crosslinks and, thus, poor digestibility. In contrast, gluten in bread drink was easily degraded after a short incubation time of 30 min and low AN-PEP activity. No significant differences of the quality parameters between treated and untreated products were found. Remarkably, bran from germinated grains was strongly enriched in nutritionally positive compounds such as dietary fiber and folates compared to native grains. Thus, wheat bran from germinated grains rendered gluten-free by treatment with AN-PEP can contribute to increasing the nutritional value of the gluten-free diet.

Keywords: Celiac disease; Gluten degradation; Prolyl endopeptidase; Wheat bran; Bread drink

Introduction

Celiac disease (CD), also called gluten-sensitive enteropathy, is an inflammatory disorder of the upper small intestine, affecting about 1% genetically predisposed individuals of the Western population. Therefore, it is one of the most frequent food intolerances worldwide. Storage proteins from wheat, rye, barley, and possibly oats, which are called gluten in the field of CD, trigger an immune response in the small intestine leading to an inflammation and ultimately to the destruction of the microvilli necessary for the absorption of nutrients. The only treatment is to maintain a strict gluten-free diet with an intake of less than 10 mg of gluten per day [1]. Dietetic gluten-free products are regulated by the Codex Alimentarius Standard as well as the Commission Regulation (EC) No 41/2009. A maximum of 20 mg gluten/kg food is allowed in order to justify a "gluten-free" claim [2,3]. In some countries, products with gluten contents between 20 and 100 mg/kg can be declared as "low in gluten".

Not only are gluten-free products more expensive than their gluten-containing counterparts, they also differ in regard to aroma, taste and texture and, most importantly, nutritional value [4]. Several studies revealed that numerous adult CD patients on a gluten-free diet show signs of poor status of vitamins (folate, B_6, B_{12}), minerals (iron, calcium), and fiber [5-7]. A logical approach to improving this situation would be to either render traditional gluten-containing raw materials gluten-free, or to increase the content of bioactive constituents in gluten-free foods by processing or fortification.

The goal of degrading gluten in gluten-containing raw materials without altering their quality can be achieved by using so-called prolyl endopeptidases (PEP) (reviewed by [8]). Unlike gastrointestinal human peptidases, these enzymes cleave peptide bonds next to proline residues, which are frequently occurring in gluten proteins, and are able to degrade gluten to CD-inactive peptides containing less than nine amino acids. Possible sources for PEP are bacteria [9], fungi [10], and germinated cereal grains [11]. Previous studies have shown that AN-PEP, an *Aspergillus niger* prolyl endopeptidase, is not only highly active towards celiac-active substrates, but also capable of completely eliminating gluten from wheat starch with contents up to 2,000 mg gluten per kg [12]. AN-PEP was initially developed as 'Brewers Clarex™' for the prevention of chill-haze in beer and the de-bittering of protein hydrolyzates. It can be applied for the production of gluten-free foods from gluten-containing raw materials [13]. A gluten-free beer introduced by the Craft Brew Alliance (Portland, OR, USA) in 2012, is brewed using traditional ingredients such as malted barley. Contained gluten is degraded by adding Brewers Clarex™ during the brewing process [14]. However, the use of AN-PEP to 'deglutenize' further foods has not been described up to now.

Bran is a food ingredient with a high content of bioactive compounds such as dietary fiber, minerals, and folic acid. As part of gluten-free foods it would, therefore, have the potential to improve the nutritional status of CD patients on a gluten-free diet. Furthermore, bran is free of cholesterol and comparatively low in calories. Bran from germinated cereals would even be more suitable for gluten-free foods, because germination of cereals under controlled conditions not only induces peptidase activity supporting gluten-degradation, but also increases the contents of valuable nutrients [15].

Beverages obtained by fermentation of cereals or cereal products have gained considerable popularity. This is not only true for beer, but also for cereal-based soft drinks. Bread drink originates from Russia

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('Kwas') and is produced by subjecting sourdough bread from wheat, rye, or oats to a non-alcoholic lactic acid fermentation for up to six months [16]. Bread drink is mainly consumed because of its health-promoting effects [17,18] and has reached a considerable sales volume. However, the product contains gluten in a concentration above 20 mg/kg and can, therefore, not be consumed by CD patients. Based on previous work, it should be possible to remove residual gluten in bread drink to enable a gluten-free claim.

Therefore, the aim of the present study was to produce gluten-free wheat bran and bread drink by using AN-PEP for complete gluten degradation. Furthermore, the content of bioactive compounds such as folate and dietary fiber in bran should be increased by germination of wheat grain. This type of food would contribute to increasing the nutritional value of the gluten-free diet.

Material and Methods

Chemicals

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

Food samples

Common wheat cv. Hermann 2011 (bran 1) and germinated common wheat cv. Hermann 2009 (bran 2, germination for 7 days at 25°C as described in [19]) were milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved yielding white flour (particle size <0.2 mm) and bran (>0.2 mm). Thus, bran 1 was from non-germinated and bran 2 from germinated wheat. Bran 3 was a commercial sample purchased in a local store. Bread drink from a German producer was used as additional sample.

AN-PEP

A commercially available proline-specific peptidase preparation from *Aspergillus niger* (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 (0.1 - 750 mg/mL). The gluten-specific peptidase activity was determined according to Schwalb et al. [19]. For comparability to previous work [12], all activity values are based on gliadin as a substrate and pH 4.0. Briefly, gliadin (50 mg) from flour of common wheat cv. Cubus [20] 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 and magnetically stirred for 150 min at 50°C. Incubated samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and gliadin degradation was monitored at 210 nm [19]. The specific peptidase activity of AN-PEP based on gliadin as a substrate was 23 U/g [12].

Gluten Degradation by AN-PEP

Wheat bran

Wheat bran samples (5 g) were mixed with AN-PEP solution (50 µL; 1 - 750 mg/mL; $1.2 \bullet 10^{-3}$ – $8.7 \bullet 10^{-1}$ U) and distilled water (20 mL). The pH value was adjusted between 1.0 and 9.0 in increments of 1.0 by adding buffer (1 mL, 0.2 mol/L; pH 1, glycine-HCl; pH 2-4, sodium acetate-acetic acid; pH 5-8, phosphate; pH 9.0, tris(hydroxymethyl) aminomethane/HCl). Vessels were closed, shaken, inserted into a water bath, incubated under continuous agitation, centrifuged (room temperature RT ≈ 20°C, 20 min, 3,760 *g*), and the residue lyophilized. The conditions were modified as follows: Incubation time, 4 - 72 h; temperature, 4, 20, 30, 40, 50, 60°C at pH 4.0, and 50°C at pH 1 - 9.

Bread drink

Bread drink (5 mL) was mixed with aqueous AN-PEP solution (50 µL; 0.1 - 10 mg/mL; $1.2 \bullet 10^{-4}$ – $1.2 \bullet 10^{-2}$ U), the mixture was vigorously shaken; the vessel was closed, and incubated in a water bath. The reaction was stopped by heating to 90°C for 10 min. The conditions were modified as follows: Incubation time, 0 - 30 min; temperature, 4, 20, 30, 40, 50, 60, 70, 80, 90°C; pH-value, 1 - 9 adjusted with buffer (1 mL) as described above.

Gluten Quantitation by ELISA

Gluten was quantitated by means of a competitive ELISA using the R5 antibody [21] (RIDASCREEN® Gliadin competitive, R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions [22]. Gliadin concentrations were converted into gluten concentrations by multiplying by a factor of two [2,22].

Protein Content and Protein Distribution of Wheat Bran

The crude protein content (N x 5.7) of wheat bran was determined by means of the Dumas method according to ICC standard 167 [23]. A TruSpec Nitrogen Analyzer (Leco, Kirchheim, Germany) was used. The quantitative distribution of Osborne fractions in bran was determined by an extraction/RP-HPLC method according to Wieser et al. [24]. Aliquots of the albumin/globulin, gliadin, and glutenin fractions were analyzed on a Thermo instrument (Thermo Electron Corp., Dreieich, Germany) using a C_{18} silica gel column (2.1 x 150 mm, 3 µm, 30 nm; Dionex, Idstein, Germany) at 60°C. Elution solvent A was 0.1% (v/v) trifluoroacetic acid (TFA), and solvent B was 0.1% (v/v) TFA in acetonitrile. The injection volumes were 15 µL for the albumin/globulin, 15 µL for the prolamin, and 20 µL for glutelin fraction. The solvent gradient was stepwise linear starting with 0-1 min 0% B, 1-11 min 0 - 20% B and 11-14 min 20 - 90% B (albumins/globulins) or 1-17 min 0-30% B and 17-23 min 30-90% B (prolamins, glutelins). The flow rate was 0.3 mL/min and the detection wavelength was 210 nm. Reference gliadin from the Prolamin Working Group (PWG-gliadin) [25] dissolved in 60% (v/v) aqueous ethanol (2.5 mg/mL) was used for calibration.

Dietary Fiber Content of Wheat Bran

The soluble, insoluble, and total dietary fiber content of untreated and AN-PEP treated wheat bran was determined according to AOAC Official Method 991.43 [26]. All samples were used in a homogenous state and measured in duplicates. A total dietary fiber assay kit (Sigma Aldrich, Steinheim, Germany) was used for digestion with α-amylase, protease, and amyloglycosidase.

Folate Content of Wheat Bran

The folate content of untreated and AN-PEP treated wheat bran was determined by means of the VitaFast® Folic Acid Kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions [27]. Homogenized sample (1 g) was digested with 10 mg chicken pancreatin in the dark at 37°C overnight (16 h) to degrade folate polyglutamates to mono- or diglutamates. Together with a folic acid medium (150 µL), digests (150 µL in different dilutions) were pipetted

into a microtiter plate coated with *Lactobacillus rhamnosus*. The growth of the microorganism depends on the supply of folic acid present in a standard or in the sample solution. Microbial growth was measured as turbidity in a microtiter plate reader (ASYS Expert 96, Biochrom Asys, Cambridge, United Kingdom) at 620 nm and compared to a standard curve with defined folate concentrations (0.16 – 1.28 µg/100 g).

Sensory Analysis of Bread Drink

Untreated and AN-PEP treated bread drink was mixed with apple juice and water (1/1/1, v/v/v) before sensory analysis according to the manufacturer's recommendation for consumption. A triangle test for similarity according to ISO 4120:2004 [28] was carried out to determine whether a perceivable sensory difference existed between the mixes containing treated and untreated bread drink. Conventional sensory profiling was carried out according to ISO 13299:2003 [29] by comparing the bread drink mix for the attributes pleasantness of aroma, taste and aftertaste as well as the intensities of the attributes bitter, acidic, and sweet on a scale from 1 to 5. All sensory experiments were carried out by a panel of 24 persons.

Statistical Analysis

Data was statistically evaluated using Microsoft Office Excel 2010 (Microsoft Corporation, Seattle, Washington, USA). The following values were applied for assessing levels of significance: $p>0.05$, insignificant; $p<0.05$, statistically significant; $p<0.01$, significant; $p<0.001$, highly significant.

Results and Discussion

Gluten degradation in wheat bran

ELISA analysis gave high gluten contents of 107,285 mg/kg, 5,335 mg/kg, and 53,333 mg/kg in bran 1, 2, and 3, respectively. The lower gluten content of bran 2 compared to the other bran samples was a result of germination, which induced endogenous peptidase activity [11] that partially hydrolyzed the gluten to about 5% to 10% of the content present in non-germinated bran. Bran is rich in minerals and folic acid as well as dietary fiber and, therefore, improves the nutritional status of foods. Conventional bran products, mostly produced from wheat or rye are often used to enrich products in dietary fiber in order to guarantee an optimal supply. Our investigations have shown that commercially available bran contains approximately 50,000 mg gluten/kg; the daily recommended consumption of at least 25 g bran [30] would thus add up to a gluten intake of 1,500 mg, which is by far more than can be tolerated by CD patients.

The treatment of bran with AN-PEP at pH 4.0 and 50°C led to a decrease of the gluten content of all samples (Table 1). The following data is based on an amount of 5 g bran. After incubation with AN-PEP (0.12 U) for 48 h, the gluten content of bran 1 was below 20 mg/kg. The same result was obtained after treatment of bran 2 with AN-PEP (0.46 U) for 24 h. The gluten content of bran 2 was even reduced below the limit of quantitation (LOQ; 10 mg gluten/kg) of the ELISA method after a longer incubation time (48 h) with a lower enzyme concentration (0.12 U). The reason was the presence of a low initial gluten concentration due to germination of the grains before milling and bran isolation (see above). Studies on gluten degradation as affected by pH and temperature showed that brans 1 and 2 were obtained gluten-free after incubation at 40-50°C and pH values between 1.0 and 4.0 or without addition of buffers (data not shown).

Unlike the first two bran samples, bran 3 was more resistant to complete gluten degradation. It was not possible to reduce the gluten content of bran 3 to less than 142 mg/kg even with a very high AN-PEP activity (0.87 U) and a long incubation time (72 h) (Table 1). According to the manufacturer's information this bran had not been treated in any specific way and was merely stored under a protective atmosphere to ensure a long shelf life despite of a fat content of 5.0 g/100 g. An Osborne fractionation of all bran samples was performed in order to see if the protein composition was considerably different (Table 2). As compared to bran 1 from minimally processed (= milled) wheat grain (glutenin content 21%), bran 3 had a considerably higher content of glutenins (36%). This indicates some kind of treatment such as excessive heating. Heat treatment can lead to incorporation of gliadins into the glutenin fraction [31,32] by thiol-disulfide interchange. In addition, heating has been shown to induce the formation of isopeptide crosslinks in glutenin [33]. Both disulfide and isopeptide crosslinks would lead to a very compact glutenin structure, which is likely to be more resistant to proteolytic cleavage compared to unmodified glutenin. This would explain the resistance of bran 3 to complete gluten degradation. Altogether, gluten degradation in wheat bran was more difficult than in wheat starch [12] due to its high initial gluten content. For example, wheat starch samples can be deglutenized at temperatures between 4 and 60°C and pH values between 1.0 and 6.0 [12], while gluten degradation in bran (bran 1 and 2) is only possible at 40 to 50°C and pH values between 1.0 and 4.0 or without addition of any buffers.

Influence of AN-PEP treatment on quality parameters of wheat bran

The crude protein content of wheat bran samples before and after AN-PEP treatment showed no significant difference (Table 3). The content of dietary fiber was only weakly affected by the treatment with AN-PEP (Table 4). The total dietary fiber content of bran 2 (untreated, 40.6%; treated, 42.2%) was considerably higher than of

AN-PEP concentration (mg/mL)	Enzyme activity ($U \cdot 10^{-1}$)	Incubation time (h)	Gluten content (mg/kg)[a]		
			Bran 1	Bran 2	Bran 3
100	1.2	24	579 ± 42^{A}	22 ± 2^{A}	904 ± 144^{A}
400	4.6	24	127 ± 3^{B}	16 ± 9^{A}	411 ± 28^{B}
100	1.2	48	15 ± 3^{C}	<LOQ[b]	215 ± 64^{C}
400	4.6	48	5 ± 0^{D}	<LOQ[b]	142 ± 3^{D}
500	5.8	72	n.d.[c]	n.d.[c]	152 ± 29^{CD}
750	8.7	72	n.d.[c]	n.d.[c]	170 ± 9^{C}

[a]Mean value of duplicate determinations ± standard deviation. Values associated with different capital letters within the same sample denote significant differences (two-sided t-test, $p<0.05$)

[b]Limit of quantitation (10 mg gluten/kg)

[c]Not determined

Table 1: Gluten content of bran samples as affected by AN-PEP concentration and incubation time. 5 g bran was incubated with 50 µL AN-PEP solution and incubated at 50 °C and pH 4.0. Initial gluten contents of bran 1, 2, and 3 were 107,285 mg/kg, 5,335 mg/kg, and 53,333 mg/kg.

Sample	Albumins / Globulins (%)[a]	Gliadins (%)[a]	Glutenins (%)[a]
Bran 1	31.3 ± 1.1^{A}	47.5 ± 0.5^{A}	21.2 ± 0.5^{A}
Bran 2	41.1 ± 0.2^{B}	40.3 ± 0.4^{B}	18.6 ± 0.6^{B}
Bran 3	29.4 ± 3.1^{A}	34.5 ± 0.5^{C}	36.1 ± 0.8^{C}

[a]Mean value of duplicate determinations ± standard deviation. Values associated with different capital letters within the same column denote significant differences (two-sided t-test, $p<0.05$).

Table 2: Content of albumins/globulins, gliadins, and glutenins (% of total extractable protein) of bran samples before treatment with AN-PEP

Sample	Protein content (%)[a]	
	Before treatment	After treatment
Bran 1	13.1 ± 0.1	13.1 ± 0.1
Bran 2	13.9 ± 0.1	13.8 ± 0.3
Bran 3	15.6 ± 0.1	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg.

Table 3: Protein content of bran samples before and after AN-PEP treatment. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Protein contents of bran 1 and bran 2 before and after treatment were not significantly different (two-sided t-test, p>0.05).

Sample	Dietary fiber (%)[a]					
	Before treatment			After treatment		
	Soluble	Insoluble	Total	Soluble	Insoluble	Total
Bran 1	3.3 ± 0.4	27.6 ± 0.2	30.9 ± 0.3	1.5 ± 0.1	24.3 ± 0.5	25.8 ± 0.3
Bran 2	1.9 ± 0.0	38.7 ± 0.9	40.6 ± 0.5	1.7 ± 0.1	40.6 ± 0.1	42.2 ± 0.1
Bran 3	3.1 ± 0.0	49.3 ± 0.5	52.3 ± 0.3	n.d.[b]	n.d.[b]	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg

Table 4: Content of soluble, insoluble, and total dietary fiber in bran samples before and after treatment with AN-PEP. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Values for insoluble and total dietary fiber were significantly higher for bran 2 compared to bran 1 (same raw material; two-sided t-test, p>0.05).

Sample	Folate content (µg/kg)[a]	
	Before treatment	After treatment
Bran 1	196 ± 21	266 ± 29
Bran 2	4534 ± 132	4233 ± 30
Bran 3	506 ± 25	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg

Table 5: Folate content of bran samples before and after treatment with AN-PEP. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Folate contents of bran 2 were significantly higher than contents of bran 1 and bran 3 (t-test, p<0.05).

bran 1 (untreated, 30.9%; treated, 25.8%). Bran 2 was obtained from germinated wheat grains. Previous work has shown that the dietary fiber content increases during germination [15], and this has been confirmed here. This is important because several studies showed that the intake of dietary fiber is insufficient, in particular among celiac patients [6,7]. This may lead to a compromised bowel function and constipation [7]. Thus, using deglutenized bran from germinated wheat grains is likely to have an additional health benefit for CD patients compared to the use of conventionally produced gluten-free wheat bran.

The total folate content of bran was analyzed by means of a VitaFast® Folic Acid Kit, a microbiological method for the quantitative determination of folic acid in accordance with international norms. Folate concentrations of bran samples before and after treatment ranged from 196 µg/kg (bran 1 before treatment) up to 4233 µg/kg (bran 2 after treatment) (Table 5). No considerable differences between treated and untreated bran samples were observed. Thus, treatment of bran with AN-PEP had no negative effects on folate, and it was obvious that bran 2 from germinated wheat was enriched in folate compared to bran 1 and 3. In summary, germination had two positive effects in this work. On the one hand it decreased the initial gluten content of bran before the addition of AN-PEP, and on the other hand it induced folate biosynthesis in the developing seed resulting in an almost 20-fold increased folate concentration compared to bran 1. This confirms previous studies on germination of wheat, in which a strong increase of different folate vitamers as well as of total folate was found in germinated wheat grain [15].

In summary, the studies on bran have shown that it is possible to render wheat bran gluten-free by peptidase treatment, even when very high initial gluten concentrations are present. An additional health effect can be obtained in the processed bran if the grains were germinated before bran isolation. This leads to an improvement of the nutritional value due to increased folate and fiber, so that gluten-free bran produced in this way can play an important role in the diet of CD patients.

Gluten degradation in bread drink

The initial bread drink had a gluten content of 82.5 mg/kg, which is in the range of many cereal-based beverages [34]. As shown in Figure 1, gluten was easily degraded in this type of food. While an AN-PEP activity of 1.2 • 10^{-4} U only led to a small reduction of the gluten content (5 g sample, 50°C, 0 - 30 min) a tenfold increase of the activity (1.2 • 10^{-3} U) decreased the gluten content to the threshold after an incubation time of 10 min and below the threshold after 30 min. A further increase of the activity by a factor of 10 (1.2 • 10^{-2} U) caused an immediate reduction of the gluten content below 20 mg/kg and a reduction below the LOQ after 10 min. After a 10 min treatment with 1.2 • 10^{-2} U, gluten was reduced under the LOQ in a very broad range of temperatures between 4 and 90°C and pH values between 1.0 and 9.0. Compared to gluten degradation in wheat bran only about 1/100 of the AN-PEP activity was required for bread drink. Furthermore, bread drink could be rendered gluten-free instantly, while at least 24 h were required in wheat bran. This difference is mainly due to (1) the lower initial gluten content of bread drink compared to wheat bran and (2) the presence of largely hydrolyzed gluten in bread drink. Compared to intact gluten, the mean molecular mass of gluten fragments in bread drink is much lower, thus requiring less peptide bonds to be cleaved by the peptidase to achieve peptides consisting of less than nine amino acids, which are no longer toxic to CD patients [35].

Sensory properties of bread drink

The manufacturer of the bread drink recommended consuming

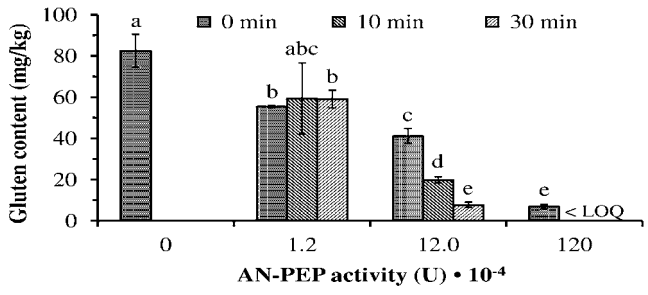

Figure 1: Gluten content (mg/kg) of bread drink as affected by AN-PEP concentration and incubation time. 5 mL bread drink were mixed with 50 µL AN-PEP solution (0.1, 1.0, 10.0 mg/mL, corresponding to 1.2 • 10^{-4}, 12.0 • 10^{-4}, and 120 • 10^{-4} U) and incubated at pH 4.0 and 50 °C for 0 to 30 min. LOQ, limit of quantitation. Values associated with different letters denote significant differences (two-sided t-test, p<0.05).

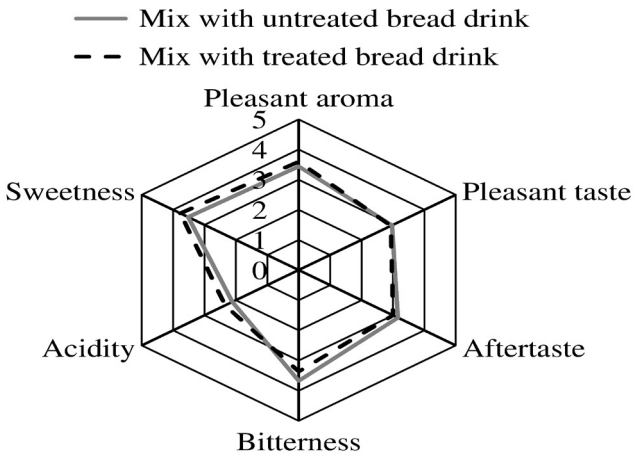

Figure 2: Sensory profiles of bread drink mixes (bread drink/apple juice/water, 1/1/1, v/v/v) containing untreated and gluten-free bread drink. 5 mL bread drink were mixed with 50 µL AN-PEP solution (10 mg/mL, corresponding to 1.2 • 10^{-2} U) and incubated at RT for 10 min. Profiles are not significantly different (p>0.05 for all attributes).

it as mixture of equal volumes of bread drink, apple juice, and water. Therefore, sensory evaluation was carried out with this mixture. A triangle test for similarity was carried out with a sensory panel of 24 individuals to detect an existing difference or similarity between the treated and untreated bread drink. This method is statistically more efficient than a duo-trio test and can be used to detect whether a difference exists in a single or in several sensory attributes. In the first part of the experiment one of the three samples was gluten-free, while one gluten-containing sample was present in the second part. Nine out of 24 panelists properly recognized the different sample. Statistical evaluation gave a type two error of β=0.10 (the likelihood of a conclusion that no distinguishable difference exists, although there is a difference). This means that less than 30% of the population mean can detect a difference between the untreated and the AN-PEP treated bread drink with a confidence level of 90%, i.e. only slight differences were present.

Conventional sensory profiling of the bread drink mix was carried out in addition to the triangle test. The panelists had to evaluate both samples separately for different attributes. Aroma, taste and aftertaste were assessed for their pleasantness, while the intensities of acidity, bitterness and sweetness had to be rated, each on a scale from 1 to 5. The results are shown in Figure 2. Although the mix containing the gluten-free bread drink was evaluated slightly more acidic and marginally less bitter than the gluten-containing mix, profiles were not significantly different (all statistical significances >0.05: pleasant aroma, 0.6; pleasant taste, 0.9; aftertaste, 0.6; bitterness, 0.5; acidity, 0.4; sweetness, 0.5). Therefore, it can be concluded that treatment of bread drink with AN-PEP for gluten degradation is possible without any adverse effects regarding its taste and aroma.

Conclusions

This study is an example that gluten-containing foods can be rendered gluten-free by enzymatic hydrolysis of gluten and gluten fragments. AN-PEP appears to be the enzyme of choice because it manages to degrade even very high gluten concentrations (≈100,000 mg/kg) in different matrices at pH values between 1.0 and 4.0 and temperatures between 40 and 50°C without any adverse effects. Strains of the genus *Aspergillus* have a food grade status, therefore, AN-PEP is suitable to be used in the production of food. The nutritional value of wheat bran can be increased by germination. In combination with enzymatic gluten degradation, products with high contents of dietary fiber and folates can be obtained that are interesting ingredients for healthy gluten-free foods. Bread drink can easily be rendered gluten-free by peptidase treatment without impacting its taste and aroma. This increases the choice of gluten-free foods for CD patients.

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