Pyrolytic acrylamide formation from purified wheat gluten and gluten-supplemented wheat bread rolls

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Recent studies have revealed different acrylamide formation mechanisms, *e.g.* from carnosine (*N*-β-alanyl-L-histidine) and aminopropionamide as additional precursors. The occurrence of acrylamide in food matrices devoid of common precursors such as meat supports an additional formation pathway. Gluten was recovered from wheat flour by water extraction. Starch, reducing sugars and amino acids were removed using α-amylase and NaCl solution and were completely absent in the purified gluten fraction. The gluten was dry heated at temperatures ranging from 160 to 240°C for 8 to 12 min and analyzed for acrylamide and cinnamic amide using liquid chromatography-tandem mass spectrometry. Acrylamide could be detected up to 3997 μg/kg gluten dry weight. Cinnamic amide was detected and unambiguously identified in the gluten samples, thus confirming the proposed formation of acrylamide from proteins. After gluten addition to bread roll dough, protein pyrolysis to form acrylamide in the complex food matrix was assessed. Contents of asparagine and reducing sugars were diminished due to the addition of the gluten. In contrast to the expectation with respect to the well-established common formation mechanism of acrylamide, it increased from 53.4 to 63.9 μg/kg (+20%), which was in good correlation with the higher proportion of gluten. As demonstrated by the *t*-test, the increase in acrylamide was significant when comparing 0 and 15% gluten addition. Additionally, cinnamic amide could be found in crusts of bread rolls. Thus, evidence for pyrolytic formation of acrylamide from wheat gluten was provided.

Keywords: Acrylamide / Bakery products / Cinnamic amide / Formation / Gluten / Protein pyrolysis

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1 Introduction

Due to the potential carcinogenic properties of acrylamide, the announcement of the Swedish National Food Authority and the University of Stockholm in April 2002 regarding findings of acrylamide in foodstuff initiated intense interest and rapid research efforts. Many studies have been accomplished to find ways to minimize the levels of acrylamide in heated products [1 – 7]. This can be reached either by modifying the processing parameters such as pH, temperature, and time of heating, or by elucidating the mechanistic pathways of acrylamide formation and eliminating precursors or intermediates. As a result of these studies, asparagine and, to a lower degree, a few other amino acids emerged as the major compounds responsible for acrylamide formation in heat-treated food products like potato chips and bakery wares in the presence of reducing sugars or carbonylic compounds [8 – 12]. Detailed mechanistic studies in model systems revealed the decarboxylated Amadori product of asparagine as the key precursor in acrylamide formation [13]. A carbonyl source is needed to activate asparagine by forming a Schiff base which decarboxylates during heating [10]. Acrolein and ammonia have also been identified as precursors of acrylamide especially in lipid-rich foods where it is formed by thermal degradation of triglycerides. The importance of these mechanisms for acrylamide formation relative to that of asparagine must independently be determined in the pertinent food [14]. However, these formation mechanisms are inadequate to explain findings of acrylamide in other heated food commodities devoid of reducing sugars but rich in protein like meat [15, 16], where the polysaccharide glycogen is rapidly transformed into lactic acid *post mortem*. This may point to additional formation mechanisms as recently revealed in other studies [17, 18]. Although the formation in a pyrolysis reaction of proteins was assumed earlier [19], detailed studies are missing

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Abbreviations: FID, flame ionization detector; **RI**, refractive index detector

so far. Model studies on peptides indicate the formation of acrylamide from proteins as hypothesized prior to our study and provide detailed mechanistic data [20]. Furthermore, investigations on different flours from wheat harvested in 2003 showed a strong correlation between crude protein content of the flour and acrylamide levels in bread rolls, pointing to an alternative pathway of acrylamide formation (unpublished).

Therefore, the objective of this study was to investigate acrylamide formation from protein pyrolysis in foodstuffs with special focus on gluten, due to its importance to bakery wares. To consider possible matrix effects, isolated gluten as well as bread rolls was included in the study. For this purpose, gluten was added to bread rolls, and acrylamide levels were determined subsequently.

2 Materials and methods

2.1 Chemicals and materials

Acrylamide (99%) was purchased from ICN Biomedicals (Eschwege, Germany), 2,3,3-d_3-labeled acrylamide (98%) was from Cambridge Isotope Laboratories (Andover, MA). Formic acid, ACN (both gradient grade), ethanol (96%), DMSO, hydrochloric acid, sodium chloride, sodium hydrochloride, sulfuric acid, ethyl acetate, methanol, glucose, fructose, sucrose, maltose, copper (II) sulfate, sodium carbonate, citric acid, potassium iodide, potassium hexacyanoferrate (Carrez I) and zinc acetate (Carrez II) were purchased from VWR (Darmstadt, Germany). Deionized water was used throughout. SPE cartridges (Isolute Multimode, 1000 mg) were obtained from IST (Hengoed, Mid Glamorgan, UK). Chem Elut cartridges for SP supported liquid-liquid extraction were purchased from Varian (Darmstadt, Germany). Enzymatic analysis kits on starch were provided by Boehringer Mannheim (Mannheim, Germany). BAN 480 L (α-amylase) for gluten pretreatment was from Novo Nordisk (Bagsværd, Denmark). Fermipan red yeast was used for bread roll dough preparation (Uniferm, Werne, Germany). Cinnamic amide as standard was synthesized, characterized, and provided by Prof. Spitzner, Institute of Chemistry, Section Bioorganic Chemistry, Hohenheim University, Germany.

2.2 Gluten purification

The preparation of gluten devoid of reducing sugars and free amino acids, especially asparagine, was a prerequisite. For this purpose, gluten was extracted from wheat (*Triticum aestivum* L.) as shown in Fig. 1. The variety used was Thasos from 2003 harvest with a high content of crude protein of 21.4% and 0.55% ash in flour purchased from the Institute of Crop Farming and Grassland Research, Hohenheim University, Germany. First, dough was prepared from 3 kg flour and 3 L water. By washing with water over a sieve, apparent starch was removed. Subsequently, the gluten fraction was treated with α-amylase solution (10 000 ppm) for 2 h at 50°C in a laboratory mixer (UM 12, A. Stephan & Söhne, Hameln, Germany). After 5 min of stirring, a rest of 10 min was allowed. To remove residual starch and degradation products like dextrine, maltose, and glucose, the gluten fraction was washed with amylase solution three times for 1 h each. Due to the higher solubility of albumins and globulins in electrolytes, washing was accordingly continued with 2% NaCl solution and the purified gluten subsequently freeze-dried. After grinding of the proteins in a Grindomix GM 200 laboratory mill (Retsch, Haan, Germany), they were washed again three times with α-amylase solution for complete elimination of sugars. Amylase residues were removed by washing with water, and the obtained gluten was freeze-dried again.

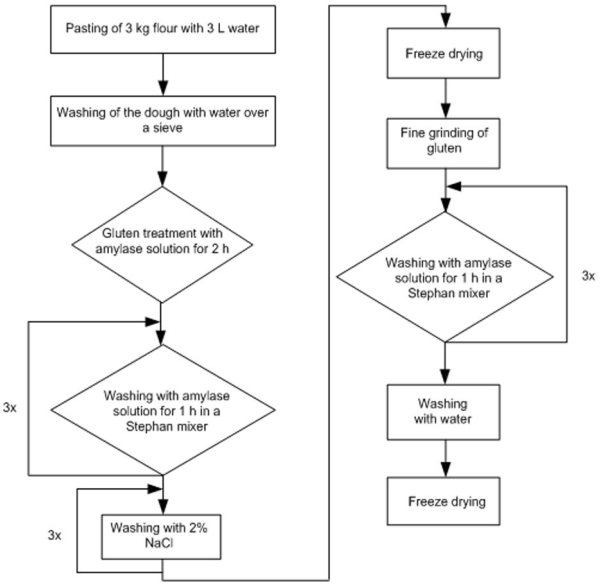

Figure 1. Extraction and purification of gluten from wheat flour.

2.3 Dry heating of gluten

The effect of dry heating on acrylamide formation was investigated by protein pyrolysis of purified gluten at different temperatures and times. For this purpose, 8 g of finely ground freeze-dried gluten was weighed into crucibles and heated at 160, 180, 200, 220, and 240°C for 6, 8, and 12 min, respectively, in triplicates in a convection oven (Euromat M8, Wiesheu, Affalterbach, Germany). After heat treatment, samples were allowed to cool and stored under vacuum at −25°C until further analyses.

2.4 Gluten addition to wheat bread rolls

Because matrix effects, water activity, changing pH values, and enzymatic activities (amylase, protease) during dough preparation and baking might influence pyrolytic protein degradation and formation of acrylamide from gluten, bread rolls were prepared from the same wheat which was used for gluten extraction, according to common manufacturing practice with the addition of 0, 5, and 15% of purified wheat gluten. In detail, three dough samples per addition were prepared from 500 g wheat flour, 300 g water, 25 g yeast, 7 g NaCl, and gluten (weight related to flour). The amount of water was corrected when gluten was added to assure constant moisture in all dough samples. After fermentation (70% relative humidity, 35°C) for 20 min, bread rolls of about 80 g each were formed (Optimat S, Eberhard Bäckereitechnik, Gräfelfing, Germany) and incubated for additional 20 min. Immediately before baking at 230°C for 15 min, pH values of the dough's were determined (691 pH meter, Metrohm, Filderstadt, Germany), and an aliquot was frosted with liquid nitrogen to inhibit further enzymatic activity of yeast and subsequently freeze-dried for the determination of reducing sugars and amino acids. Baked bread rolls were allowed to cool at room temperature and then separated into crusts and crumbs. The latter have been shown to be devoid of acrylamide [5, 21]. Their weight was determined before and after crust separation for recalculation of total acrylamide contents. Crusts were freeze-dried and stored at −25°C under vacuum until analysis.

2.5 Determination of starch

Gluten was separated from starch that may generate glucose after heating [22] to exclude acrylamide formation from reducing sugars. Therefore, the presence of residual starch was enzymatically tested according to the manufacturer's instructions. For this purpose, 2.5 g of sample material was weighed in a 50-mL Erlenmeyer flask. After the addition of 20 mL DMSO and 5 mL hydrochloric acid (8 N) samples were incubated for 30 min at 60°C in a water bath under stirring and rapidly cooled to room temperature. Subsequently, 5 mL of sodium hydrochloride (8 N) were added for neutralization. The solution was adjusted to 50 mL using citrate buffer (pH 4.6) and filtered for the enzymatic test. The reaction was monitored by photometric determination (Cary 100 Cone, Varian, Palo Alto, CA) of reduced nicotinamide adenine dinucleotide phosphate at 340 nm.

2.6 Determination of reducing sugars

Reducing sugars were determined using LC with refractive index detection (HPLC-RI) [23]. Samples were ground, and 10 g was weighed in a 250-mL Erlenmeyer flask together with 100 mL of 60% ethanol, homogenized with an ultra turrax, and sonicated in a water bath at 70°C for 20 min. Hot ethanolic extraction was performed to prevent sugar degradation by yeast. HPLC-RI analysis was carried out using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation Software, a model G1379A degasser, a model G1312A binary gradient pump, a model G1313A autosampler, a model G1316A column oven, and a model G1362A refractive index detector. The separation was performed using a Polyamine II column (250 × 4.6 mm; 5 µm) (YMC Europe, Schermbeck, Germany), equipped with an NH_2 guard column (4.0 × 3.0 mm) (Phenomenex, Torrance, CA) operated at 35°C. The injection volume was 20 µL, isocratic elution was performed with a mobile phase of 60% ACN at a flow rate of 1 mL/min and a total run time of 15 min. Sugars were detected with a refractive index detector operated at 35°C. Individual compounds were quantified using a calibration curve of the corresponding standard compounds ranging from 5 µg/L to 5 g/L.

In addition to the chromatographic method, total reducing sugars were determined using the Luff-Schoorl method [24]. For this purpose, 10 g of sample material was weighed in a 250-mL volumetric flask and 150 mL of water was added. After suspension and homogenization of the sample material with an ultra turrax, 5 mL of Carrez solution was pipetted, adjusted to 250 mL with water, and filtered. Aliquots of 25 mL of the filtrate and 25 mL of Luff solution (45.7 g citric acid, 144.2 g sodium carbonate, and 16 g copper (II) sulfate made up with water to 1 L) were transferred in a 250-mL Erlenmeyer flask and heated at 100°C. After 10 min, samples were rapidly cooled on ice water, and 3 g of potassium iodide and 25 mL of H_2SO_4 (25%) were added. Titration was performed with sodium thiosulfate until a yellowish color occurred. After addition of a few drops of starch solution as an indicator, titration was continued until the blue color disappeared. Quantification was performed using a calibration curve obtained with standard solutions. Results were calculated as maltose, which is the major sugar in fermented dough.

2.7 Determination of amino acids

Amino acids, especially asparagine, were determined by GC and flame ionization detection (GC-FID) using a clean up and derivatization kit (EZ:faast, Phenomenex). For this purpose, 12.5 g of fine ground sample material was weighed in a 100-mL Erlenmeyer flask and extracted with 50 mL of 45% ethanol for 30 min. After centrifugation for 15 min at 4000 rpm, 3 mL of the supernatant was filtered through a 0.45-µm syringe filter and 400 µL of the filtrate was subjected to clean up and derivatization. For correction of analyte losses during sample preparation, 100 µL of the

internal standard norvaline was pipetted together with the effluent in glass sample preparation vials and slowly passed over the sorbent tips filled with ion exchanger. Subsequently, washing solution (200 μL of isopropanol/water) was passed over the tips and the sorbent material was subsequently eluted with 200 μL of the eluting medium (NaOH/water/isopropanol/methylpyridine) into the sample vials. After completion of the clean up, derivatization was performed with 50 μL of chloroform/2,2,4-trimethylpentane/alkylchloroformiate for 2 min. Additionally, 100 μL of chloroform/2,2,4-trimethylpentane was added and allowed to react for 1 min. After washing of the derivative with 0.1 M HCl (100 μL), the upper layer was transferred in a pointed amber glass autosampler vial, and 2-μL aliquots were injected into the gas chromatograph. GC analysis was carried out with a Chrompack CP 9001 (Chrompack, Middleburg, The Netherlands) equipped with a FID operated at 250°C with a spilt ratio at 1 : 15. Helium was used as the carrier gas. The oven temperature was increased from 110 to 320°C within 7 min followed by a 1-min isothermal hold. The FID was operated at 320°C. External standard procedure was used for quantification.

2.8 Determination of acrylamide

Acrylamide was determined as recently published [21]. In brief, samples were ground to a fine powder and extracted, together with d_3-acrylamide as an internal standard, with 100 mL of water in an ultrasonic water bath at 40°C. After Carrez precipitation and filtration, a clean up using Isolute Multimode cartridges was performed to remove interfering compounds. Due to the usually low acrylamide contents in bakery wares, a concentration step was applied using Chem Elute cartridges and ethyl acetate as the eluent. After evaporation of the organic solvent and dissolution in water, samples were subjected to LC-MS/MS analyses using an Agilent HPLC 1100 series as described above coupled online to a Bruker (Bremen, Germany) Esquire 3000+ IT mass spectrometer fitted with an ESI source. Data acquisition and processing was performed using Esquire Control software. Separation was carried out with a Hypercarb column (100 × 2.1 mm; 5 μm) (Thermo Hypersil, Dreieich, Germany), equipped with a C18 guard column (4.0 × 3.0 mm) (Phenomenex) operated at 30°C. The mobile phase was 1% ACN/0.05% formic acid v/v in water (eluent A) at a flow rate of 0.2 mL/min, and a total run time of 10 min. The mass spectrometer was operated in the positive ion mode. Nitrogen was used both as the drying and as nebulizing gas. Helium was used as the collision gas for CID at a pressure of 4.0×10^{-6} mbar. Signals at *m/z* 72.3 (acrylamide) and *m/z* 75.3 (deuterium-labeled acrylamide) were isolated with a peak width of *m/z* 0.6. For the transitions *m/z* 72.3 > *m/z* 55.5 and *m/z* 75.3 > *m/z* 58.5 the fragmentation amplitude was set at 1.55 and 1.50 V, respectively. Masses were recorded using multiple reaction monitoring. For quantitation the signals at *m/z* 55.5 (*m/z* 58.5) were used, while signals at *m/z* 44.5 (*m/z* 45.5) served for qualification.

2.9 Determination of cinnamic amide

Cinnamic amide was extracted from the samples (5 g) with 50 mL of water in an ultrasonic water bath at 50°C for 10 min. Subsequently, samples were purified by Carrez clarification and filtration, and then subjected to LC-MS/MS analysis. The HPLC system was the same as described above using a Luna C18 (150 × 3.0 mm; 3 μm) equipped with a C18 guard column (4.0 × 3.0 mm) (both from Phenomenex) operated at 60°C. The mobile phase consisted of H_2O/MeOH/formic acid (40/60/0.05, v/v/v, isocratic). The flow rate was 0.25 mL and total run time was 10 min. Injection volume was 50 μL and peak confirmation was carried out by comparison of the retention time with an external standard (4.4 min). The transition observed was *m/z* 148 > *m/z* 131 (loss of NH_3).

3 Results and discussion

3.1 Gluten purification

The objective of the present study was to investigate acrylamide formation from protein pyrolysis with special focus on gluten because of its importance to bakery wares. Therefore, low molecular precursors like asparagine and reducing sugars had to be removed from the gluten, which was achieved by several washing steps combining α-amylase and NaCl solution. The purified and dried gluten was subsequently analyzed for amino acids, reducing sugars, and starch, respectively.

Determination of starch after DMSO extraction and analysis using an enzymatic test kit with a limit of detection of about 90 μg/g sample material confirmed that no residues are left, even after doubling the sample weight. Glucose could simultaneously be determined in the sample blank additionally to the HPLC-RI analysis. No change of the extinction in any of the extracts was observed.

Reducing sugars were determined using an HPLC-RI method after hot ethanolic sample extraction [23]. Glucose, fructose, maltose, and sucrose, from which reducing sugars might be formed during heat treatment, could not be found in any of the gluten samples. In addition, the Luff-Schoorl method was applied, confirming the negative results of the HPLC-RI method. The limit of detection was approximately 15 μg/g sample material.

The amino acid precursors, in particular asparagine, were removed using water and an NaCl solution during gluten

pretreatment. Gluten was analyzed for free amino acids by GC-FID after derivatization. Asparagine could not be detected in any of the samples, whereas only the lipophilic amino acids tyrosine, tryptophane, and phenylalanine were found at trace levels (<1 μg/g purified gluten each), which may be explained by their lower water solubility. Thus, gluten purification led to a substrate, which was unambiguously devoid of low molecular acrylamide precursors.

3.2 Dry heating of gluten

To verify the hypothesized formation of acrylamide by protein pyrolysis under different time/temperature conditions, freeze-dried gluten was heated at 160, 180, 200, 220, and 240°C for 6, 8, and 12 min, respectively. As shown in Fig. 2, acrylamide was formed by heating purified gluten devoid of low molecular precursors up to 3997 μg/kg. The proposed formation pathway, an electrocyclic domino reaction [25], is shown in Fig. 3. R_1 and R_2 represent further amino acids in the peptide, which can react similarly to alanine and phenylalanine. Alanine is supposed to be the key precursor of acrylamide, which is formed by a simultaneous sequence of electrocyclic processes (domino reaction) without contribution of an external carbonyl source. Such reactions are well known from the synthesis of olefins by pyrolysis of carboxylic acid esters or xanthogenates [19]. A structural requirement for the reaction described here is a β-proton present in the amino acid adjacent to alanine, which is transferred to the carbonyl oxygen of alanine and, after tautomerization, to the carboxamide group of acrylamide. The content of alanine next to a β-proton containing amino acid varies with the type of protein, with approximately 4.4% in gluten [26]. According to this pathway, further reaction products resulting from amino acids other than alanine should be generated in the heated gluten together with acrylamide. Cinnamic amide derived from protein-bound phenylalanine was found to be one of these products. Therefore, the occurrence of this degradation product in heated gluten was investigated to confirm the novel pathway of acrylamide formation. As shown in Fig. 4, cinnamic amide could be detected and unambiguously identified in dry heated gluten by its mass spectrum, thus supporting the suggested reaction mechanism. These findings clearly indicate an alternative formation route for acrylamide by thermal degradation of alanine containing peptides and proteins. This is in agreement with an earlier study [17] reporting the formation of acrylamide from carnosine (*N*-β-alanyl-L-histidine). The formation routes are not entirely comparable because carnosine contains β-alanine instead of alanine. In that study two possible pathways for the formation of acrylamide from this dipeptide were presented, first through hydrolysis of the peptide bond with the release of β-alanine followed by its subsequent deamination and rearrangement to acrylamide, and secondly through elimination of β-alanine amide and further deamination. Contrary to thermal carnosine degradation in which β-alanine is the key amino acid, the involvement of an electrocyclic domino reaction (Fig. 3) upon heating of alanine containing proteins to yield acrylamide and cinnamic amide was demonstrated in our study.

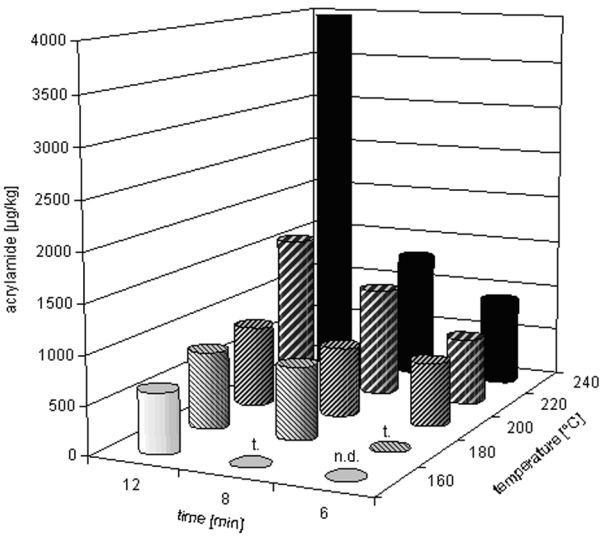

Figure 2. Acrylamide in dry gluten heated at different time-temperature regimes (n.d., not detected; t., trace levels).

Previous investigations on the presence of acrylamide in potato chips and bread had revealed a strong influence of the time-temperature regime [27–29]. Maximum acrylamide formation was found at temperatures between 180 and 200°C, while higher temperatures resulted in a decrease, probably due to degradation processes. According to these results, at lower temperatures prolonged heating times were

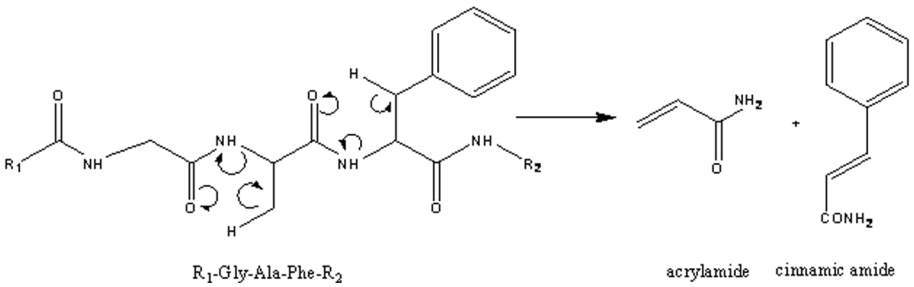

Figure 3. Proposed formation of acrylamide and cinnamic amid in protein pyrolysis (schematic).

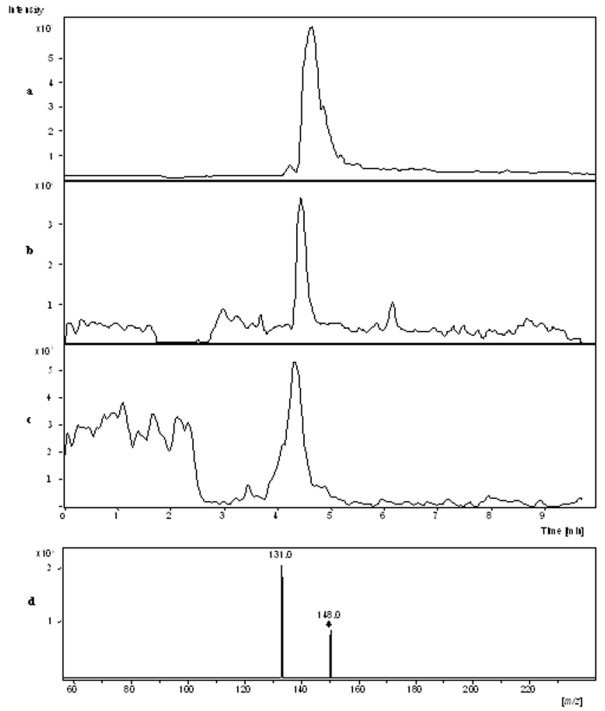

Figure 4. LC-MS/MS chromatograms (*m/z* 131) of cinnamic amide. (a) Reference compound, (b) extracted from dry heated gluten (240°C, 12 min), (c) extracted from bread rolls spiked with gluten (15%), (d) mass spectrum of cinnamic amide in dry heated gluten.

required to generate acrylamide from gluten, whereas high temperatures facilitated protein pyrolysis and acrylamide formation (Fig. 2). Degradation was not observed in our study, which may be attributed to short heating times. Compared to the formation from asparagine and reducing sugars, this pathway appeared to require higher temperatures for acrylamide formation. Acrylamide contents were comparable to other dry heated food matrices like flour, where acrylamide contents of about 3000 μg/kg were found [30, 31]. Correspondingly, carnosine was shown to have acrylamide-generating efficiency similar to asparagine/glucose in former studies [17]. Therefore, the proposed mechanism of acrylamide formation cannot be neglected when considering measures for acrylamide reduction.

3.3 Gluten addition to wheat bread rolls

Although acrylamide was generated upon heat treatment of freeze-dried gluten, matrix effects like water activity, pH-shift, enzymatic activities, and contribution of other ingredients might influence protein pyrolysis in more complex food matrices, in particular in bread and bread rolls. Therefore, bread roll dough was prepared according to industrial practice and spiked with 0, 5, and 15% of purified gluten. The pH values, controlled immediately before baking, were constantly in the range of 5.4 to 5.5. Asparagine and reducing sugars were determined in the dough samples. The latter were expressed as sum of total fructose, glucose, sucrose, and maltose. As shown in Fig. 5, both precursors decreased when gluten was added in increasing amounts, which was ascribed to a dilution effect. Considering the well-established formation mechanism based on reducing sugars and asparagine, a decrease of the acrylamide contents would be expected upon heating. Instead, acrylamide contents increased from 53.4 to 58.8 and 63.9 μg/kg (20% increase), respectively, depending on the amount of added gluten (5 and 15%), thus supporting the proposed pathway. In contrast to our findings, gluten addition was found to decrease the acrylamide content in crackers in an earlier study [31]. However, the gluten used in the previous study only consisted of 80% protein, and the chemical composition of the remainder constituents, probably containing acrylamide decreasing agents like cysteine, was not specified. Additionally, crusts of bread rolls were tested for cinnamic amide. As can be seen from Fig. 4, this key degradation product could unambiguously be detected in the crust, even when no gluten was added. Therefore, the formation of acrylamide from protein pyrolysis in bakery products can be deduced.

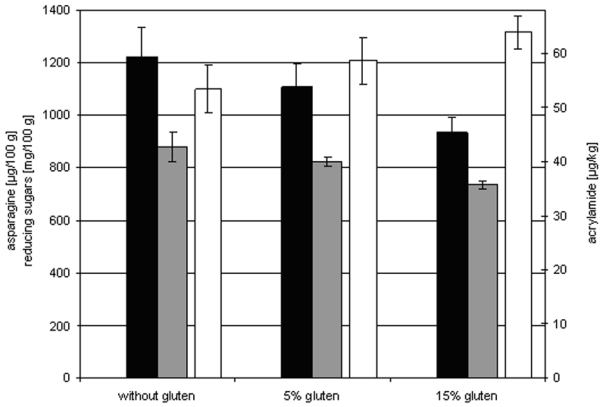

Figure 5. Asparagine (black bars) and reducing sugars (grey bars) in dough and acrylamide (white bars) in bread rolls after addition of different amounts of gluten.

To exclude random errors, a *t*-test was performed with bread rolls containing 0 and 15% of gluten supplement to establish a significant difference ($\alpha = 0.05$) in acrylamide contents of the baked products. The *t*-test was calculated from the mean of the determinations and the corresponding SD. It could be shown that acrylamide contents significantly exceeded that of the unspiked bread rolls.

4 Concluding remarks

The proposed pathway of acrylamide formation by an electrocyclic domino reaction during protein pyrolysis could be

confirmed by detection of both acrylamide and cinnamic amide in dry heated gluten devoid of low molecular precursors. The contribution of the established mechanism to acrylamide formation in more complex food matrices like bakery products was tested by gluten addition to common bread rolls. Whereas the content of the low molecular precursors decreased, probably due to a dilution effect, the acrylamide levels significantly increased.

The relevance of this novel formation pathway on the acrylamide content in different food matrices needs to be further assessed. In particular, the influence of gluten composition with respect to the alanine content and of different production parameters like pH, water activity, and protease activity of the flour is a matter of our current investigations. Furthermore, from our results it becomes also evident that technological and plant-breeding measures exclusively focusing on low molecular precursors will only lead to a partial reduction of acrylamide in bakery products.

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