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Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a heterocyclic aromatic amine belonging to a class of mutagens found in cooked food. Phenylacetaldehyde and 2-phenylethylamine, thermal degradation products of phenylalanine, were found to react with creatinine to form PhIP. To identify possible intermediates in the model system and in fried meat an aldol addition between phenylacetaldehyde and creatinine was made. The aldol addition product (2-amino-1-methyl-5-(1′-hydroxy-2′-phenylethyl)-imidazol-4-one) was then dehydrated to get the aldol condensation product (2-amino-1-methyl-5-(2′-phenylethenyl)-imidazol-4-one). These two chemically synthesised compounds were then used as standards. The aldol addition product could not be found in the model system and also not in fried meat. In contrast the aldol condensation product was identified, by liquid chromatography in combination with mass spectrometry and UV spectroscopy, in the model system and in fried meat. The data show that phenylacetaldehyde and the aldol condensation product of phenylacetaldehyde and creatinine are very important intermediates in the reaction mechanism to form PhIP.

Keywords: PhIP; Model system; Heterocyclic aromatic amines; Formation; Reaction mechanism

1. Introduction

The heterocyclic aromatic amine PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) is a mutagenic and potential carcinogenic compound (Sugimura, 1997). It is formed during heating of food (meat, fish) that contains creatine, free amino acids and carbohydrates. Heterocyclic aromatic amines are the results of very complex reactions. Besides creatin(in)e and amino acids, monosaccharides are involved via the Maillard reaction, as reviewed by Skog (1993). Model systems are used to simplify the meat matrix. By that the high number of parallel reactions are reduced and the studying of the reactions of interest is simplified. Some researchers used a meat juice model system to study the formation of heterocyclic amines under defined but more realistic conditions (Arvidsson, van Boekel, Skog, Solyakov, & Jägerstad, 1999; Skog, Solyakov, & Jägerstad, 2000). The group of Felton used mixtures of amino acids, creatine and glucose simulating the composition of six different kinds of meat. With these dry-heated model systems the formation of heterocyclic amines in meats was simulated (Pais, Salmon, Knize, & Felton, 1999). A total of 10 mutagenic heterocyclic amines have been identified to be carcinogenic in rodents. Among these, PhIP, generally the most abundant in addition to MeIQx with normal cooking procedures, induces mammary and colon carcinomas in rats in a clear dose-dependent manner (Ito et al., 1997). PhIP was first isolated and identified from fried ground beef by the group of Felton et al. (1986). The formation of PhIP in a model system with glucose was first reported by Shioya, Wakabayashi, Sato, Nagao, and Sugimura (1987). Övervik, Kleman, Berg, and Gustafsson (1989) were successful in producing PhIP in a model system without sugar. Skog and Jägerstad (1991) showed that by adding glucose to phenylalanine and creatine more PhIP was formed than without. They also demonstrated an inhibiting effect of glucose in excessive molar proportions versus the other precursors.

The mechanisms for the formation of heterocyclic aromatic amines have not been clarified in detail. A few postulated mechanisms are described in literature (Jones & Weisburger, 1989; Yaylayan, Pare, Laing, & Sporns,

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1990). Kikugawa (1999) found that free radical intermediates, pyrazine cation radicals and carbon-centred radicals, generated in the Maillard reaction of sugars/amino acids, were involved in the production of mutagenic and carcinogenic imidazoquinoxaline-type heterocyclic amine mutagens. Kato, Harashima, Moriya, Kikugawa, & Hiramoto (1996) reported that the heated model mixture of glucose/glycine or glucose/glycine/creatinine generated unstable carbon-centred radicals. Jägerstad et al. (1983) described that through Maillard reaction followed by Strecker degradation pyrazines, pyridines and aldehydes are formed. Creatine, pyrazines or pyridines and aldehydes are assumed to condense to form IQ (imidazoquinoline) compounds. Pearson, Chen, Gray, and Aust (1992) proposed two different pathways for the formation of imidazoquinoline and imidazoquinoxaline meat mutagens. They found a contribution of free radicals, which are formed by the Maillard reaction, the Amadori rearrangement and a sugar fragmentation.

Felton's group investigated the formation of PhIP by heating phenylalanine and creatine and showed that the phenyl ring from phenylalanine is incorporated intact. They also showed that the 3-carbon atom and the amino nitrogen from phenylalanine are incorporated into PhIP. By using labelled creatine they found that the 1-nitrogen, the methyl-carbon, and the amino-nitrogen from creatine are each incorporated into PhIP (Taylor, Fultz, Morris, Knize, & Felton, 1988). From these experiments it can be assumed that the carbon atoms of phenylalanine form a part of the pyridine moiety and that creatine forms the imidazol part.

The investigation of the mechanism of the formation of PhIP was the primary aim in this study. Murkovic, Weber, Geiszler, Fröhlich, and Pfannhauser (1999) postulated a reaction mechanism for the formation of PhIP. They described a degradation of phenylalanine to phenylacetaldehyde, a dimerisation of phenylacetaldehyde and a nucleophilic addition as possible reaction steps. To confirm this mechanism phenylacetaldehyde and the condensation product of phenylacetaldehyde and creatinine had to be identified in the model system and in fried meat. For a positive identification the postulated intermediates had to be chemically synthesised and characterised first.

2. Materials and methods

2.1. Chemicals

All chemicals and solvents were of HPLC or analytical grade. Water was distilled twice and additionally purified with activated carbon (Millipore, Bedford, USA). All solvents, for example acetonitrile, dichloromethane (DCM), diethylene glycol (DEG) and methanol, were purchased from Merck (Darmstadt, Germany). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3-methyl-9H-pyrido[2,3]indole (MeAαC) were obtained from Toronto Research Chemicals (Toronto, Canada). L-phenylalanine was purchased from Merck, DL-phenylalanine-^{15}N from Isotec Inc. (Miamisburg, Ohio, USA), phenylacetaldehyde and phenylacetic acid from Sigma-Aldrich (Vienna, Austria) and creatinine, 2-phenylethanol, 2-phenylethylamine and styrol from Fluka (Buchs, Switzerland). For the solid phase extraction an Oasis MCX cartridge (3 cc/60 mg or 6 cc/150 mg, 30 μm) of Waters (Milford, Massachusetts, USA) was used.

2.2. Model systems

For the determination of thermal degradation products of phenylalanine, 40 mg phenylalanine were dry heated. The samples were heated in Headspace-Vials at 150 °C for 10 min using a thermostated heating block with electronically controlled temperature. Then the vial was cooled down to 30 °C and after piercing the septum the fiber was exposed to the headspace at 30 °C for 10 min.

For the standard model system the precursors, creatinine (26 mg) and phenylalanine (40 mg) were dissolved in equimolar ratio in 25 ml diethylene glycol and heated to 200 °C for 60 min. After the reaction the model system was cooled to room temperature immediately by putting the reaction flask into a water/ice mixture. With this model system the identified thermal degradation products of phenylalanine were tested if they form PhIP in a reaction with creatinine. Therefore they were applied in an equimolar ratio to 26 mg creatinine (28 mg phenylacetaldehyde, 31 mg 2-phenylethylamine, 32 mg phenylacetic acid, 30 mg 2-phenylethanol, 27 mg styrol). This model system was also used to identify the intermediate reaction products of the PhIP formation. To prepare PhIP-^{15}N the same standard model system was used, but with ^{15}N labelled phenylalanine instead of phenylalanine.

A modification of the standard model system was used to determine if PhIP is formed at room temperature. Therefore phenylalanine and creatinine were solved in DEG and stirred for 2 weeks.

To determine the kinetics of the formation of PhIP and of the condensation product of phenylacetaldehyde and creatinine standard model systems were made and purified after 10, 20, 30, 40, 50 and 60 min. To determine the kinetics of the formed phenylacetaldehyde a dry heated model system as described earlier was used. Forty milligrams phenylalanine and 26 mg creatinine were heated for 15, 30, 45 and 60 min.

2.3. Purification

An aliquot of the standard model system (250 μl) was dissolved in 3 ml 0.1 M HCl and spiked with 0.11 μg

MeAαC as internal standard. For HPLC analysis, the clean-up was done by a SPE method. The cartridges were conditioned with 2 ml MeOH and 2 ml water. Then they were loaded with the spiked sample, washed with 2 ml 0.1 M HCl, 2 ml 100% methanol and 2 ml 40% methanol with 5% ammonium hydroxide (NH_4OH). The elution was done with 95% methanol with 5% ammonium hydroxide. The eluent was evaporated and the residue dissolved in methanol for HPLC analysis. For preparation of PhIP-^{15}N, phenylalanine labelled with ^{15}N, was used. For LC–MS analysis the reaction mixture was extracted with DCM as described previously (Murkovic et al., 1999) and further purified as described earlier.

2.4. Preparation of the aldol reaction product

The reaction was done in a round-bottom two-neck flask with temperature control and a dropping unit. Creatinine (0.01 mol) was dissolved in 10 ml methanol and 0.005 mol of a methanolic potassium hydroxide solution (15%) were added. As creatinine has only one reactive methylene group, it was applied equimolar to the aldehyde. Phenylacetaldehyde (0.01 mol) was dissolved in 10 ml methanol and added dropwise by stirring and cooling the reaction mixture to 15 °C. Then the mixture was stirred for additionally two hours at room temperature (Becker et al., 1999). The clean-up was done by dilution of the batch with 50 ml water, neutralising with acetic acid and removal of interfering reaction products by extraction with DCM. After filtration the aqueous phase was ready for HPLC analysis.

2.5. Preparation of the aldol condensation product

The preparation of the condensation product of phenylacetaldehyde and creatinine was done by elimination of water of the addition product (Becker et al., 1999). The aqueous phase of the aldol reaction was evaporated and 1.2 g of the residue were dissolved in 2 ml phosphoric acid (85%). The reaction was done at 160 °C for 90 mins. The viscous reaction mixture was diluted in 5 ml water and after filtration analysed by HPLC.

2.6. Isolation of synthesised reaction products of phenylalanine and creatinine

For HPLC analysis of the reaction mixtures a Hewlett Packard 1100 liquid chromatograph with a diode array detector and a mass spectrometer, equipped with a LiChrospher RP-select B (5 μm, 125×4 mm) and a precolumn LiChroCART 4–4 (LiChrospher 60 RP-select B, 5 μm) was used. The column was eluted with a mobile phase of 90% methanol/acetonitrile/acetic acid/water (8/14/76/2) with a pH set to 5.0 with ammonium hydroxide and 10% methanol. The flow rate was set to 1.0 ml/min and the UV-absorption was monitored at 254 nm. MS analysis was performed using a HP quadrupole mass spectrometer equipped with an electrospray interface. Drying nitrogen was heated to 350 °C and the drying gas flow was 10 l/min. The capillary voltage was set to 4000 V and the positive mode was used. For data acquisition, the mass spectrometer operated over a mass range of m/z 100–450 and for special identification the SIM (single ion monitoring) mode was used (m/z 234 for the aldol addition product and m/z 216 for the aldol condensation product of phenylacetaldehyde and creatinine).

For the preparative chromatography of the aldol reaction product the reaction mixture was evaporated to dryness and redissolved in 10 ml mobile phase. The reaction mixture of the condensation product was diluted with 5 ml water and then used for chromatography. For the preparative isolation of the two intermediate reaction products the same method without a mass spectrometer was used. The preparative HPLC was performed with a LiChrospher RP-select B (5 μm, 125×8 mm, Pannosch, Vienna, Austria) with an injection volume of 100 μl. The UV-absorption was monitored at 254 nm and the flow was 3.5 ml/min. For the removal of the mobile phase the collected fraction was preconcentrated by Oasis MCX cartridges (150 mg/6 cc). To prepare the sorbent for use, the cartridges were conditioned with 5 ml MeOH and 5 ml water. Then the cartridges were loaded with 25 ml of the fraction, washed with 5 ml 0.1 M HCl and 5 ml 100% methanol. The elution was done with 95% methanol with 5% ammonium hydroxide. After evaporation of the eluent, the residue was ready for NMR analysis.

2.7. HPLC analysis of PhIP and intermediates

For HPLC analysis of PhIP, 10 μl of the extracts were injected into a Hewlett Packard 1100 liquid chromatograph with a fluorescence detector, equipped with a LiChroCART Superspher 60 RP-select B (5 μm, 125×2 mm, Merck, Darmstadt, Germany) and a precolumn LiChroCART 4–4 (LiChrospher 60 RP-select B, 5 μm). The column was eluted with a mobile phase of methanol/acetonitrile/acetic acid/water (8/14/76/2) with a pH set to 5.0 with ammonium hydroxide (A) and methanol (B). The gradient was as follows. From 0 to 10 min B was increased from 0 to 80%, and from 10 to 12 min B was kept at 80%. The column was then reconditioned using the initial solvent composition. The flow rate was set to 0.4 ml/min and the fluorescence of the effluent was monitored at 370 nm for PhIP ($\lambda_{ex} = 316$ nm) and 395 nm for MeAαC ($\lambda_{ex} = 345$ nm). For HPLC analysis of intermediates in model systems and fried meat the same method as for PhIP was used. The detection was performed by diode array and mass spectrometry. The UV-absorption was monitored at 254 nm. For mass spectrometry

detection the parameters as described above were used. The identification was done by comparing the retention times with those of the preparative produced compounds. Additionally the UV-spectra were compared with that of the chemically synthesised substance.

2.8. GC analysis of thermal degradation products

This technique was used for the investigation of thermal degradation products of phenylalanine and for the determination of phenylacetaldehyde in dry heated model systems and in fried meat. The analytes were concentrated by headspace extraction on a Carboxen/Polydimethylsiloxane (Carboxen/PDMS) fibre from Supelco (Vienna, Austria). The analysis of the enriched compounds was done by GC–MS. For the identification of the volatile components the "Wiley 275″ library was used. Additionally, linear temperature programmed retention indices (RI) were calculated according to the equation of van den Dool and Kratz (1963) and compared to RI data from a retention index database (Farkaš, Le Quéré, Maarse, & Kovác, 1994; Siegmund, Derler, & Pfannhauser, 2001).

For the analysis a Hewlett Packard G 1800 A GCD System with a HP5 GC column (30 m×0.25 mm id × 0.25 mm film thickness) was used. The initial temperature was −30 °C, the final temperature was 280 °C and the rate was 10 °C/min. Helium was used as carrier gas (constant flow off, pressure 0.54 bar) and the injection mode was splitless.

2.9. NMR analysis

The products obtained after HPLC purification were dissolved in CD_3OD. The ^{13}C-NMR (125.69 MHz; 499.82 MHz) and ^{1}H-NMR (75.47 MHz; 300.13 MHz) spectra were measured on a Varian INOVA-500.

2.10. Identification of intermediates in meat and in model system

For the isolation of the aldol condensation product in the standard model system the same purification as for PhIP was used. For the purification 1 ml of the model system was applied to the SPE cartridge and the residue of the eluate was dissolved in 100 µl methanol. For the identification of the condensation product in meat, pork was fried as described previously (Murkovic, Friedrich, & Pfannhauser, 1997). In brief, a piece of pork chop (boneless) was fried for 25 min on both sides simultaneously using a Teflon-coated heating device. The size of the meat was 10 cm×15 cm, with a thickness of 1 cm. No salt, oil or spices were added. After cooling down, the fried meat was homogenised with a mixer (B400, Büchi, Flawil, Switzerland). About 4 g were suspended in 25 ml MeOH at room temperature on a magnetic stirrer for 3 h. After extraction and filtration the solvent was evaporated and the residue dissolved in 5 ml MeOH and directly analysed with HPLC and mass spectrometry.

3. Results

3.1. Quality assurance

In general, all samples were run in duplicate. Duplicate values varied by about ±5%. For the analysis of PhIP some additional parameters were measured. The recovery of PhIP and MeAαC was determined to be about 90%. This was found by some spiking experiments. Additionally MeAαC, which could not be found in the samples, was used as internal standard and PhIP was corrected for incomplete recovery. The HPLC showed linearity in the range used.

3.2. Thermal degradation products of phenylalanine

The thermal degradation products of phenylalanine were identified by GC–MS. Potential precursors for the formation of PhIP are identified in Fig. 1. They all contain the phenyl group and two carbon atoms attached to it having different functionalities (styrol, phenylacetaldehyde, 2-phenylethylamine, 2-phenylethanol, phenylacetic acid). Using phenylacetaldehyde or 2-phenylethylamine in the model system instead of phenylalanine PhIP was formed. The reaction of creatinine with the others (styrol, 2-phenylethanol, phenylacetic acid) did not lead to the formation of PhIP. The identification of PhIP was done by comparing the retention times and the fluorescence spectra with that of an authentic standard. By analysing with LC/MS, a positive identification was achieved by comparing the retention times and the mass spectra. In all model systems the

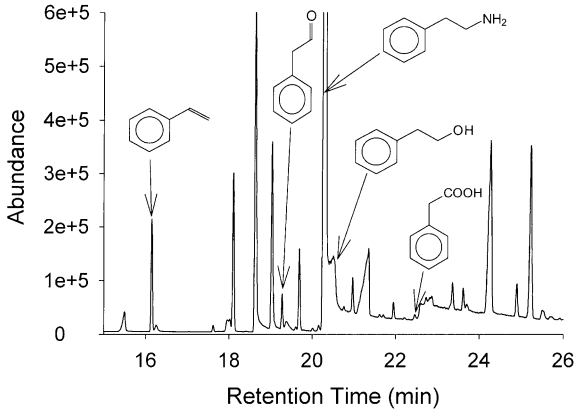

Fig. 1. Headspace analysis of products formed after heating of phenylalanine to 150 °C for 10 min. The identified structures are from left to right: styrol, phenylacetaldehyde, 2-phenylethylamine, 2-phenylethanol, phenylacetic acid.

degradation products were applied equimolar to creatinine. In those experiments where PhIP was formed, the quantities were compared. Fig. 2 shows that in the standard model system with phenylalanine and creatinine 9.5±0.5 μg PhIP were produced. In the model system with phenylacetaldehyde and creatinine the amount was much higher, 13.7±0.7 μg PhIP were formed. In contrast, the quantity of the produced PhIP was very low in a model system with 2-phenylethylamine and creatinine as reactants (1.2±0.1 μg).

3.3. Other model systems

Besides the model reactions that were already described above, a few additional reactions were carried out. In a model system with creatinine and phenylalanine that was performed at room temperature for two weeks, no formation of PhIP occurred.

In the model reaction with DL-phenylalanine-^{15}N and creatinine, 10% of the formed PhIP had no ^{15}N incorporated. 77% of the formed PhIP had a mass that was 1 unit higher than natural PhIP, showing that one ^{15}N was incorporated. Furthermore 13% of the formed PhIP incorporated two ^{15}N, the mass was two units higher than natural PhIP.

3.4. Preparation of standard compounds

In the postulated isomer two optical active carbons were formed, resulting in two diastereomers that can be separated by HPLC (data not shown) both of them having the molecular mass of 233 which corresponds to the aldol addition product. This was confirmed by NMR analysis after preparative chromatography. The ratio was 3:1. The structure of the aldol addition product (2-amino-1-methyl-5-(1′-hydroxy-2′-phenylethyl)-imidazol-4-one) was also confirmed by NMR analysis, it is shown in Fig. 3. The ^{1}H-NMR data are as follows. The chemical shifts indicate the N-methyl of creatinine (imidazole) at 3.1 ppm, a $-CH_2$ (benzylic) at 2.8 ppm, the secondary alcohol at 4.1 ppm and the aromatic hydrogens at 7.1–7.3 ppm. The ^{13}C-NMR data also confirmed the structure of the aldol addition product. The chemical shifts indicate the N-methyl at 32 ppm, the $-CH_2$ (benzylic) at 42 ppm, the secondary alcohol at 73 ppm and the aromatic carbon atoms at 128, 130, 131 and 140 ppm.

After dehydration of the aldol addition product at 160 °C the first intermediate disappeared. The aldol addition product was converted quantitatively. Besides some side products there was one substance with the expected molecular mass of 215 dominating the chromatogram. It is postulated to be the dehydration product of the aldol addition product which is equivalent to the aldol condensation product of phenylacetaldehyde and creatinine. Because of the instability, this product was destroyed after preparative HPLC during the purification and could not be confirmed by NMR measurements.

Normally the α,β-unsaturated aldehyde (2-amino-1-methyl-5-(2′-phenylethyliden)-imidazol-4-one) is formed through the aldol reaction (Streitwieser, Heathcock, & Kosower, 1994). It is stabilised because of the conjugated system between the C–C double bond and the carbonyl double bond. But the C–C double bond increases the ring strain of the five-membered ring. Thus another structure (2-amino-1-methyl-5-(2′-phenylethenyl)-imidazol-4-one) is probable through electron rearrangements. Both isomeres are shown in Fig. 4. In this structure the C–C double bond is formed between the creatinine part and the phenyl group of the molecule. There is no resonance stabilisation between the double bond and the carbonyl group. The ring strain is less than in the α,β-unsaturated form because the C–C double bond has moved away from the five-membered ring. But because the double bond is conjugated to the phenyl group there is an additional resonance stabilisation in this region of the molecule. Therefore this structure should be more stable than the α,β-unsaturated form. This structure was confirmed by interpretation of UV measurements. Creatinine has a UV maximum at a wavelength of 242 nm. Toluol, which was measured to

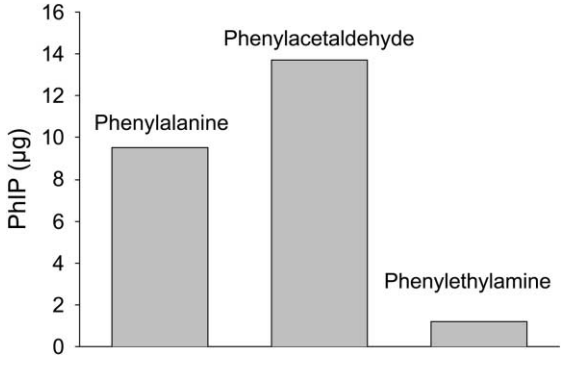

Fig. 2. Formation of PhIP in a model system using creatinine and a second precursor as indicated in the graph.

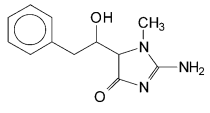

Fig. 3. Aldol addition product (2-amino-1-methyl-5-(1′-hydroxy-2′-phenylethyl)-imidazol-4-one) of creatinine and phenylacetaldehyde.

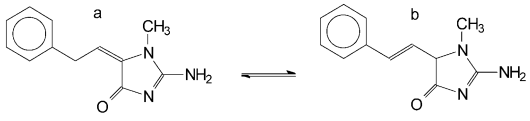

Fig. 4. Two possible isomeres of the aldol condensation product of creatinine and phenylacetaldehyde. (a) 2-Amino-1-methyl-5-(2′-phenylethyliden)-imidazol-4-one, (b) 2-amino-1-methyl-5-(2′-phenylethenyl)-imidazol-4-one.

determine the UV spectra of the aromatic ring with a methyl group in the side chain, has a UV maximum at 261 nm. An additional conjugated double bond results in a bathochromic shift of 30 nm. An aromatic ring with a conjugated double bond has a UV maximum of about 290–295 nm (Gottwald & Heinrich, 1998). For the aldol condensation product of phenylacetaldehyde and creatinine two UV maxima were measured. One UV maximum was 295 nm, which corresponds to the aromatic ring and a conjugated double bond. A second UV maximum was determined at 252 nm. It corresponds to the creatinine ring. The bathochromic shift of 10 nm is due to an attachment of a hydrocarbon residue.

3.5. Identification of intermediate products

The identification of the aldol condensation product in model system and in fried meat was done by comparing the retention times after separation with HPLC, the molecular mass and UV spectra with the synthesised intermediate. The chromatograms of the reaction product of creatinine and phenylalanine in the model system and of the synthesised substance are shown in Figs. 5 and 6. The identification of the aldol condensation product in the model system through the retention times was unequivocal as with the MSD as well with the DAD. The additional identification by the UV spectrum was also unobjectionable.

The identified intermediate in the model system was also found in fried pork meat which was fried for 25 min using a heating device. The identification of the aldol condensation product was positive by means of molecular mass and UV spectra. The chromatograms of synthesised reaction product of creatinine and phenylacetaldehyde and analysis of the reaction product in fried meat are shown in Figs. 7 and 8. UV spectra of synthesised reaction product of creatinine and phenylacetaldehyde, the reaction product in the model system and the reaction product in fried meat are shown in Fig. 9.

3.6. Kinetics

The kinetics of phenylacetaldehyde, the condensation product as intermediate and PhIP were determined for the first 60 min of the reaction. Phenylacetaldehyde was

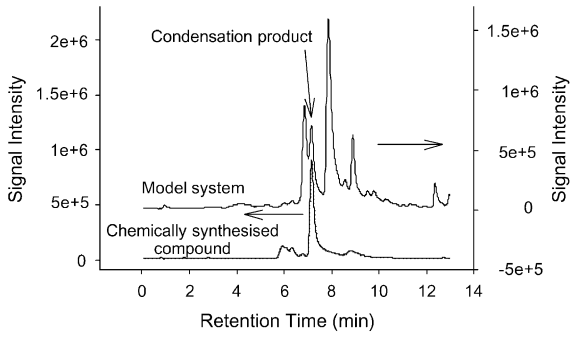

Fig. 5. MSD chromatogram (SIM) of synthesised reaction product of creatinine and phenylacetaldehyde and analysis of the reaction product in the model system.

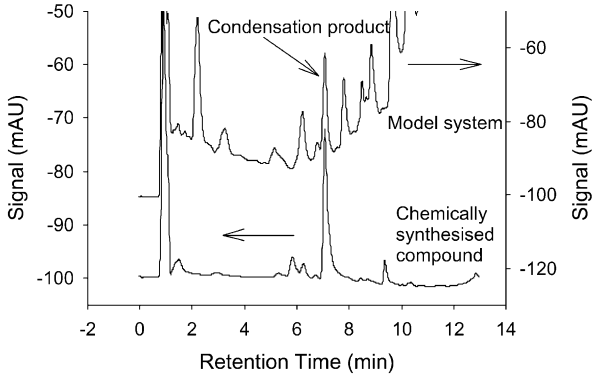

Fig. 6. DAD chromatogram of synthesised reaction product of creatinine and phenylacetaldehyde and analysis of the reaction product in the model system.

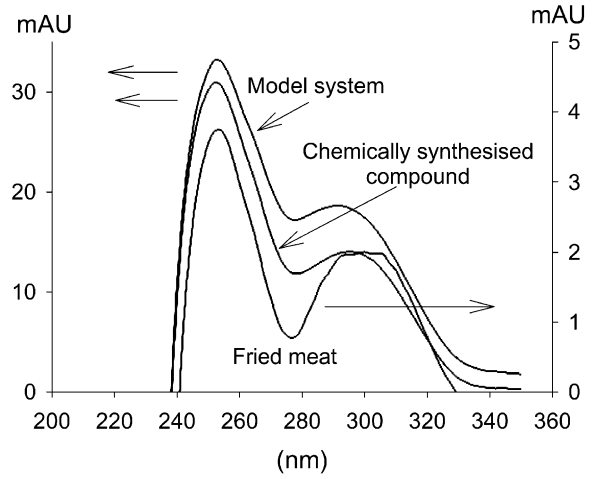

Fig. 7. MSD chromatogram (SIM) of synthesised reaction product of creatinine and phenylacetaldehyde and analysis of the reaction product in fried meat.

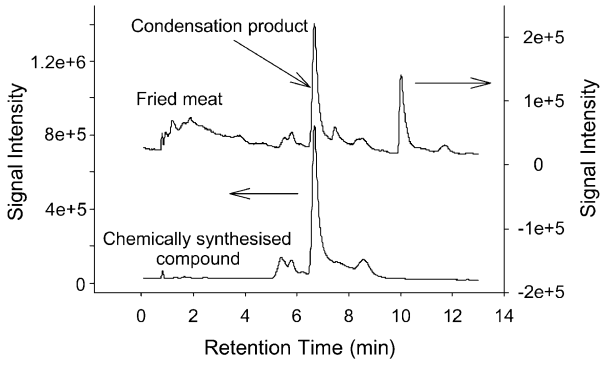

Fig. 8. DAD chromatogram of synthesised reaction product of creatinine and phenylacetaldehyde and analysis of the reaction product in fried meat.

measured by SPME/GC–MS after dry heating of phenylalanine and creatinine. The aldol condensation product and PhIP were determined in the standard model system at 200 °C. As phenylacetaldehyde was measured in a different model system, it cannot be compared directly with the condensation product and PhIP. The kinetics are shown in Fig. 10. Phenylacetaldehyde and the intermediate were formed very fast in the model reactions. Their amounts reached a maximum at 10 min with a following decrease. PhIP increased linearly until 40 min with only a slight further increase.

4. Discussion

The result, that a mixture of phenylacetaldehyde and creatinine produced more PhIP than a standard model system, supports the finding that phenylacetaldehyde plays a key role in the mechanism of the formation of PhIP. In a previous study it was postulated, that phenylacetaldehyde is formed by a thermal degradation of phenylalanine (Murkovic et al., 1999). In the model reaction of phenylacetaldehyde and creatinine the first step of the reaction mechanism, formation of phenylacetaldehyde,

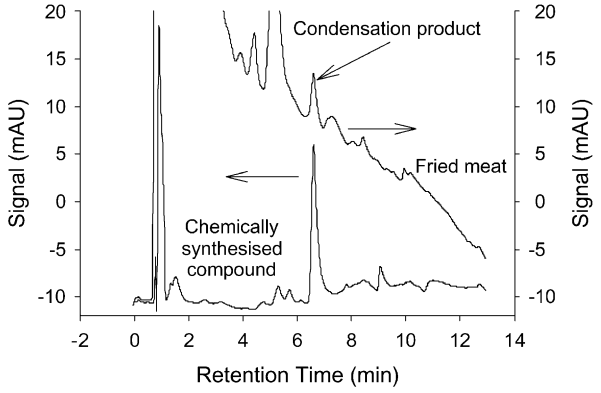

Fig. 9. UV spectra of synthesised reaction product of creatinine and phenylacetaldehyde, the reaction product in the model system and the reaction product in fried meat.

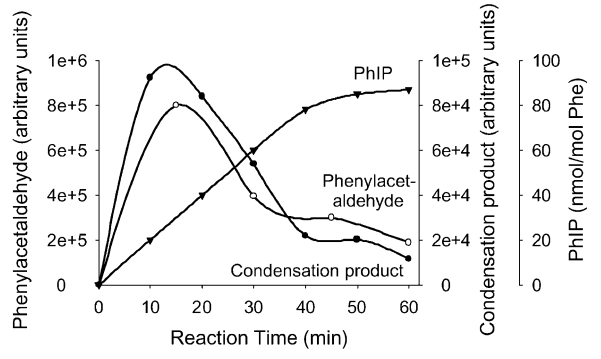

Fig. 10. Formation of phenylacetaldehyde, condensation product and PhIP in model systems.

is not necessary. Therefore more PhIP was formed. A second reason is the high amount of phenylacetaldehyde in the model system. The standard model reaction yields only a small amount of phenylacetaldehyde. As can be seen in Fig. 1 a lot of different products are formed by the degradation of phenylalanine.

In a model system with creatinine and phenylalanine that was performed at room temperature for 2 weeks, no formation of PhIP occurred. This is an additional indication that the degradation of phenylalanine to phenylacetaldehyde is necessary for the formation of PhIP. The energy in this model reaction is too low for the degradation. Manabe, Kurihara, Wada, Tohyama, and Aramaki (1992) got similar results. A slight increase in the reaction temperature to 37 and 60 °C respectively did not result in the formation of PhIP. At these low temperatures PhIP was formed by adding carbohydrates (e.g. glucose) to this model system. In this case phenylacetaldehyde was formed by the Strecker degradation of phenylalanine (Mevissen & Baltes, 1983).

In a further experiment the aldol addition product of creatinine and phenylacetaldehyde was heated in DEG at 200 °C for 60 min. PhIP was formed in this reaction (data not shown). This confirms that the aldol addition product is an intermediate in the course of the formation of PhIP. In this reaction the nitrogen of the pyridine moiety of PhIP must come from the creatinine part of the aldol addition product. The origin of the nitrogen in the pyridine ring is not clearly defined. At least part of it could be derived from the nitrogen in creatinine, possibly the amino group. This was also confirmed by the formation of PhIP in a reaction with phenylacetaldehyde and creatinine where no additional nitrogen is present.

It was shown that in the model reaction with DL-phenylalanine-^{15}N, 10% of the formed PhIP had no ^{15}N incorporated. The nitrogen of phenylalanine was not incorporated into PhIP. Seventy-seven percent of the formed PhIP had one atom of ^{15}N incorporated. Furthermore 13% of the formed PhIP incorporated two ^{15}N. This means, that the amino group of creatinine is labile and possibly exchanged by the amino group of phenylalanine. These results show that the largest amount of the pyridine nitrogen has its origin in phenylalanine. If this nitrogen is not available, the nitrogen of creatinine can also be inserted in PhIP.

The kinetics of phenylacetaldehyde, the condensation product of phenylacetaldehyde and creatinine and PhIP were determined in model systems without water. Phenylacetaldehyde was measured after dry heating of phenylalanine and creatinine. PhIP and the condensation product were determined in a standard model system, which was done by heating of phenylalanine and creatinine in DEG. Highest amounts of phenylacetaldehyde and of the condensation product were formed during the first 20 min of the reaction. After the thermal degradation of phenylalanine, the reaction between

phenylacetaldehyde and creatinine followed immediately. Phenylacetaldehyde and the condensation product were then decreasing while PhIP was increasing continually. Arvidsson, van Boekel, Skog, and Jägerstad (1997) got same results for PhIP. They heated creatinine, glucose, amino acids and carnosine in a water based model system and found a first-order model for the formation of PhIP. Skog et al. (2000) found that dry heating favoured PhIP formation, while aqueous heating favoured other heterocyclic amines.

4.1. Mechanism

The mechanism of formation of PhIP is illustrated in Fig. 11. The first reaction step of the formation of PhIP is the thermal degradation of phenylalanine (1). Through SPME/GC–MS phenylacetaldehyde (2) was identified as degradation product in a dry heated model system of phenylalanine and creatinine. When the reaction was done in DEG, phenylacetaldehyde could not be detected because the solvent masked a great range of the chromatogram. Phenylacetaldehyde was also identified in fried meat by SPME/GC–MS (data not shown). Besides a thermal degradation, a Strecker degradation of phenylalanine to phenylacetaldehyde is also possible because of the presence of carbohydrates in meat (Baltes & Mevissen, 1988). Adamiec, Rössner, Velisek, Cejpek, and Savel (2001) oxidised phenylalanine with either potassium peroxodisulfate or glyoxal at 100 °C for 1 h. They found that phenylacetaldehyde is one of the major degradation products through this oxidative decarboxylation. Jones and Weisburger (1989) also described a thermal degradation of an amino acid, L-threonine, that yields aldehydes such as acetaldehyde that reacts with creatinine to form aminomethylimidazol-4-one mutagens.

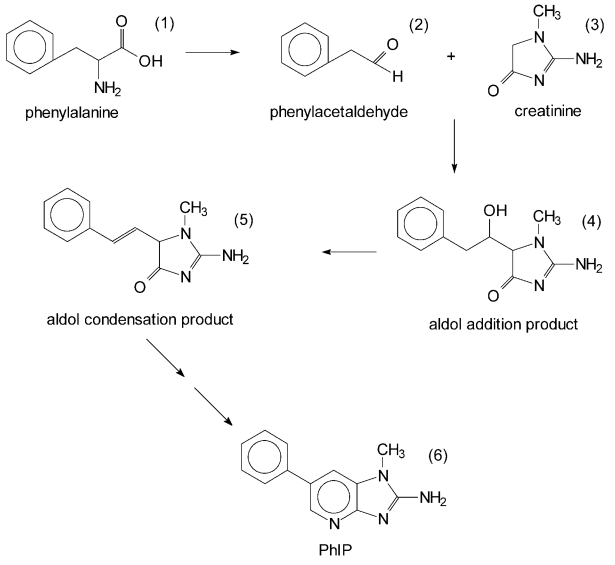

Fig. 11. Proposed mechanism of formation of PhIP in the model system and in fried meat.

The second reaction step in the formation of PhIP is an aldolisation between phenylacetaldehyde (2) and creatinine (3). The C-5 of creatinine reacts with phenylacetaldehyde in a nucleophilic addition and subsequent dehydration to form the condensation product (5) as an intermediate. The first intermediate is the aldol addition product (4). However this reaction product could not be detected neither in the model system nor in fried meat. This reaction product was not expected to occur, because of the high temperatures applied in the model system and in meat. Even the chemical synthesis of the aldol addition product of phenylacetaldehyde and creatinine resulted very easily in a dehydration and formation of the aldol condensation product. For that reason the reaction was carried out at temperatures below 20 °C. Even at these low temperatures a small amount of the condensation product was formed.

In the model reaction and also during frying of meat the aldol addition product (4) was dehydrated immediately. Therefore this intermediate reaction product could not be detected. In contrast, the aldol condensation product (5) of phenylacetaldehyde and creatinine was identified in both, the model system and the fried meat. The detection was achieved by comparing the retention time, the molecular mass and the UV spectrum with that of the synthesised standard. The exact position of the double bond could not be determined. Because of different stabilities and interpretation of UV spectra, the structure which is shown in the mechanism is suggested to be the favoured one. The α,β-unsaturated aldol condensation product is rearranged to a structure that has a conjugated double bond to the phenyl ring.

The final steps of a possible reaction mechanism could be the formation of a Schiffs base between the creatinine part of the condensation product (5) and a compound with an amino group. Phenylalanine could be one possibility, but 2-phenylethylamine could also act as reactant. This compound was already identified as a major pyrolytic degradation product of phenylalanine. Another possible reaction partner could be creatinine. In a model reaction with phenylacetaldehyde and creatinine, PhIP was produced without an additional nitrogen source. In this model system the nitrogen of the pyridine ring has its origin in the creatinine molecule. PhIP is then formed by the cyclisation to the pyridine ring, an aromatisation and a loss of several groups. A reaction involving free radicals is a plausible possibility for the splitting off.

The postulated reaction mechanism fits also with the results that were found by Murkovic et al. (1999). They made some NMR experiments using ^{13}C-labelled phenylalanine as reaction partner of creatinine and showed that the carbon atoms of phenylalanine form a part of the pyridine moiety. The mechanism does not fit exactly with the results of Felton and Knize (1990) but it fits with the experiments that were made by Murkovic et al.

(1999). They found in contrast to Felton's group that no ^{13}C-labelled PhIP is found when the carboxyl carbon of phenylalanine is labelled. This suggests that the carbon dioxide resulting from decarboxylation of phenylalanine does not further contribute to the formation of PhIP. The reaction mechanism fits with the experiments by Felton and Knize (1990), where they showed that creatinine forms the imidazol part of PhIP and the phenyl ring from phenylalanine is incorporated intact.

5. Conclusion

In this study part of the mechanism of formation of PhIP was elucidated by identification of intermediate reaction products. Phenylacetaldehyde was confirmed as a thermal degradation product of phenylalanine and important intermediate in the mechanism. Furthermore the condensation between phenylacetaldehyde and creatinine was found to be the key reaction in the formation of PhIP. The validity of the reaction mechanism was confirmed by identification of phenylacetaldehyde and the aldol condensation product of phenylacetaldehyde and creatinine as well in model systems as in fried meat. For a confirmation of the whole reaction mechanism the final intermediate that leads to PhIP has to be characterised. By clarifying the structure of this intermediate it is possible to identify the final reaction steps. Especially a possible participation of a Schiff's base reaction is of interest. Further work has also to be done to clarify the participation of free radical reactions.

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