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Effect of amino acids on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in creatinine/phenylalanine and creatinine/phenylalanine/4-oxo-2-nonenal reaction mixtures

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ABSTRACT

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) formation in mixtures of creatinine, phenylalanine, amino acids and 4-oxo-2-nonenal was studied, to analyse the role of amino acids on the generation of this heterocyclic aromatic amine. When oxidised lipid was absent, cysteine, serine, aspartic acid, threonine, asparagine, tryptophan, tyrosine, proline, and methionine increased significantly ($p < 0.05$) the amount of PhIP formed in comparison to the control. When lipid was present, only the addition of methionine, glycine, and serine increased significantly ($p < 0.05$) the amount of PhIP produced, while histidine, cysteine, lysine, tryptophan, tyrosine, and alanine reduced significantly ($p < 0.05$) PhIP. These results may be a consequence of the different competitive reactions that occur. Thus, in the absence of lipids, thermal decomposition of the amino acids produced reactive carbonyls that converted phenylalanine into phenylacetaldehyde as a key step in the formation of PhIP. When oxidised lipid was present, amino acids competed with phenylalanine for the lipid, and amino acid degradation products were formed, among which alpha-keto acids seemed to play a role in these reactions. These results suggest that PhIP can be produced by several alternative reaction pathways from all major food components, including amino acids and lipids, in addition to carbohydrates.

1. Introduction

The formation of heterocyclic aromatic amines (HAAs) in foods is a concern for food chemists, nutritionists, and toxicologists because of their potential mutagenic/carcinogenic properties (Busquets et al., 2013; Damasius, Venskutonis, Ferracane, & Fogliano, 2011; Gibis & Weiss, 2012; Nakai & Nonomura, 2013; Turesky & Le Marchand, 2011; Zur Hausen, 2012). Nowadays, more than 25 different HAAs have been identified in foods, and their relative formation is greatly influenced by food composition and cooking methods (Busquets et al., 2013). Among all of them, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is one of the most abundant HAAs. It is typically found in amounts up to 35 ng/g (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2011), but there are reports of higher levels, especially in fried and barbecued chicken (Solyakov & Skog, 2002).

At present, PhIP is considered to be mainly produced from phenylalanine, creati(ni)ne and carbohydrates as a by-product of the Maillard reaction (Murkovic, 2004; Wong, Cheng, & Wang, 2012; Zochling & Murkovic, 2002). PhIP is produced in several steps, among which the Strecker degradation of phenylalanine into phenylacetaldehyde plays a major role (Cheng et al., 2008). For that reason, lipid oxidation products, which have been shown to be efficient Strecker aldehyde producers (Hidalgo & Zamora, 2004), also produce PhIP to a significant extent (Zamora, Alcon, & Hidalgo, 2012a; Zamora, Alcon, & Hidalgo, 2013). Furthermore, any other reactive carbonyl compound that might produce the Strecker degradation of phenylalanine into phenylacetaldehyde should be considered as a potential inducer of the formation of PhIP when creati(ni)ne and phenylalanine are present and reaction conditions are appropriate.

Among major food components, the thermal decomposition of amino acids is known to produce carbonyl compounds (Rodante, 1992; Ross, 2013). Therefore, these carbonyl compounds might

Abbreviations: AlaKA, pyruvic acid; CRN, creatinine; CysKA, mercaptopyruvic acid; GluKA, α-ketoglutaric acid; GlyKA, glyoxylic acid; HAAs, heterocyclic aromatic amines; IleKA, 3-methyl-2-oxovaleric acid; LeuKA, 4-methyl-2-oxovaleric acid; MetKA, α-keto-γ-methylthiobutyric acid; MRM, multiple reaction monitoring; ON, 4-oxo-2-nonenal; Phe, phenylalanine; PheKA, phenylpyruvic acid; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TyrKA, 4-hydroxyphenylpyruvic acid; ValKA, 3-methyl-2-oxobutyric acid.

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also be contributing to PhIP formation, although to the best of our knowledge no previous reports have described a contribution of amino acids to PhIP formation. On the other hand, a recent study has suggested that amino acids can contribute to the removal of PhIP by forming PhIP–amino acid adducts (Kataoka, Miyake, Saito, & Mitani, 2012).

In an attempt to clarify the role of amino acids on PhIP formation, this investigation describes the formation of PhIP in mixtures of creatinine, phenylalanine, and amino acids. In addition, the same reaction mixtures were also studied in the presence 4-oxo-2-nonenal, which was previously shown to be a very efficient PhIP producer (Zamora, et al., 2012a; Zamora et al., 2013). The study of these last reactions has allowed the analysis of the role of amino acids in systems containing reactive carbonyls, which are efficient inducers of PhIP formation.

2. Materials and methods

2.1. Materials

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was purchased from Toronto Research Chemicals (North York, ON, Canada). 4-Oxo-2-nonenal was prepared from 2-pentylfuran according to Shimozu, Shibata, Ojida, and Uchida (2009).

The commercial α-keto acids used in this study were: glyoxylic acid (GlyKA, α-keto acid derived from glycine), pyruvic acid (AlaKA, α-keto acid derived from alanine), 3-methyl-2-oxobutyric acid (ValKA, α-keto acid derived from valine), 4-methyl-2-oxovaleric acid (LeuKA, α-keto acid derived from leucine), 3-methyl-2-oxovaleric acid (IleKA, α-keto acid derived from isoleucine), α-keto-γ-methylthiobutyric acid (MetKA, α-keto acid derived from methionine), mercaptopyruvic acid (CysKA, α-keto acid derived from cysteine), phenylpyruvic acid (PheKA, α-keto acid derived from phenylalanine), 4-hydroxyphenylpyruvic acid (TyrKA, α-keto acid derived from tyrosine), and α-ketoglutaric acid (GluKA, α-keto acid derived from glutamic acid). All these chemicals, as well as the other chemicals employed in this study, were of the highest available analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO) or Merck (Darmstadt, Germany).

2.2. Formation of PhIP in creatinine/phenylalanine/amino acid, creatinine/phenylalanine/amino acid/4-oxo-2-nonenal, and creatinine/phenylalanine/α-keto acid reaction mixtures

A solution of creatinine (10 μmol) and phenylalanine (10 μmol) in 500 μL of 0.3 M sodium phosphate buffer, pH 8, was treated, or not (control), with amino acids (10 μmol) and heated at 200 °C in closed test tubes for 1 h. After cooling (5 min at room temperature and 10 min at −20 °C), 100 μL of the reaction mixture were diluted with 50 μL of internal standard solution (1.29 mg of caffeine in 5 mL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate), and 850 μL of a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate. PhIP was determined by LC–MS/MS.

Reaction mixtures containing 4-oxo-2-nonenal were treated similarly and the lipid (10 μmol) was added at the beginning of the experiment together with creatinine and phenylalanine.

Reaction mixtures containing α-keto acids (10 μmol) were treated similarly and the α-keto acid was added in the place of the amino acid.

2.3. PhIP determination

Samples were analysed using an Agilent liquid chromatography system (1200 Series) consisting of binary pump (G1312A), degasser (G1379B), and autosampler (G1329A), connected to a triple-quadrupole API 2000 mass spectrometer (Applied Biosystems, Foster City, CA) using an electrospray ionisation interface in positive ionisation mode (ESI^+). Compounds were separated on a Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column from Agilent. As eluent, a 30:70 mixture of 0.2% formic acid in acetonitrile and 4 mM ammonium acetate was used. The mobile phase was delivered at 0.5 mL/min in isocratic mode. Mass spectrometric acquisition was performed by using multiple reaction monitoring (MRM). The nebuliser gas (synthetic air), the curtain gas (nitrogen), and the heater gas (synthetic air) were set at 45, 25, and 50 (arbitrary units), respectively. The collision gas (nitrogen) was set at 5 (arbitrary units). The heater gas temperature was set at 500 °C and the electrospray capillary voltage to 5.5 kV. The fragment ions in MRM mode were produced by collision-activated dissociation of selected precursor ions in the collision cell of the triple quadrupole and selected products were analysed with the second analyser of the instrument. Three transitions were acquired for the identification of both PhIP and the IS.

To establish the appropriate MRM conditions for the individual compounds, the mass spectrometric conditions were optimised using infusion with a syringe pump to select the most suitable ion transitions for the target analytes. Precursor and product ions used for confirmation purposes and operating conditions were described previously (Zamora et al., 2012a). The 225.0 → 210.1 and 195.2 → 138.0 transitions for PhIP and caffeine, respectively, were used for quantification purposes in this study.

2.4. PhIP quantification

Quantification of PhIP was carried out by preparing five standard curves of this compound in 400 μL of 0.3 M sodium phosphate buffer, pH 8.0, and following the whole procedure described above. For each curve, seven different concentration levels of PhIP (0–2 nmol) were used. PhIP content was directly proportional to the PhIP/IS area ratio ($r > 0.997$, $p < 0.0001$). The limit of detection (LOD), defined as the lowest sample concentration that could be detected with a signal-to-noise ratio (S/N) greater than three (Hidalgo, Alaiz, & Zamora, 2001), was 0.005 nmol. The limit of quantitation (LOQ), defined as the lowest concentration that could be quantitated with a precision less than 15%, was 0.01 nmol.

2.5. Carbonyl compound formation in heated amino acids

A solution of the tested amino acid (10 μmol) in 500 μL of 0.3 M sodium phosphate buffer, pH 8, was heated at 200 °C in a closed test tube for 1 h to study the formation of carbonyl compounds. Formation of carbonyl compounds was evaluated with 2,4-dinitrophenylhydrazine according to the method of Zamora, Alaiz, and Hidalgo (1997), which was slightly modified. Briefly, 200 μL of the heated amino acid solution were treated with 2 mL of 2,4-dinitrophenylhydrazine [a 0.4% (w/v) solution in 2 N HCl] and heated for 10 min at 100 °C. After cooling, the reaction mixture was treated with 1.1 mL hexane, stirred for 3 min, and centrifuged at 2000*g* for 5 min. The carbonyl content was calculated from its peak absorption at 370 nm, using a molar absorption coefficient (ε) of 22,000 M^{-1}.

2.6. Statistical analysis

All data given are mean ± SD values of at least three independent experiments. Statistical comparisons among different groups were made using analysis of variance. When significant *F* values were obtained, group differences were evaluated by the Tukey test (Snedecor & Cochran, 1980). Statistical comparisons were carried out using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.