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Characterization of a peptide adduct formed by *N*-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), a reactive intermediate of the food carcinogen PhIP

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Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a member of a class of compounds known as the heterocyclic amines (HCAs) that are formed in meat during cooking. It is a multi-organ carcinogen in rodents forms adducts and with DNA and protein. Although protein adducts are not thought to be involved in cancer development, they may be useful as internal dosimeters of PhIP exposure and bioactivation. Towards the goals of characterizing the adducts formed in humans and the development of an assay for quantitation of adduct levels, we have characterized a peptide adduct formed by the putative genotoxic metabolite, *N*-acetoxy-PhIP. A model peptide with the internal sequence Leu–Gln–Lys–Cys–Pro–Tyr, which is homologous to a potential target sequence for HCAs in human serum albumin, was reacted with *N*-acetoxy-PhIP and an adduct was identified and further characterized by LC–ESI-MS/MS. *N*-acetoxy-PhIP is covalently bound to the peptide via cysteine and the exocyclic amino group of PhIP. Future work is needed to establish if this adduct is formed and is stable in vivo in humans following exposure to PhIP.

Keywords: Heterocyclic amine; PhIP; Protein; Adduct; Structure; Mass spectrometry

1. Introduction

Two-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a heterocyclic amine (HCA) which is formed in relatively high concentrations during cooking of meat (Layton et al., 1995). PhIP is a potent mutagen in bacteria and mammalian cell genotoxicity assays (Thompson et al., 1987) and has been shown to cause tumors in the colon, prostate, and breast following high dose exposures in rats (Ito et al., 1991; Shirai et al., 1997). Indeed, some epidemiology studies have linked dietary HCA exposure to cancer in these organs in humans (reviewed by Sinha (2002)).

Metabolic activation of PhIP, thought to be mediated primarily through cytochrome P4501A2, followed by *N*-acetyltransferase and sulfotransferase activation (Buonarati et al., 1990; Boobis et al., 1994; Turesky et al., 1998), forms reactive intermediates that can form covalent adducts with DNA and protein (Fig. 1). Protein adducts are formed after exposure to chemical carcinogens and provide a quantitative measure of the internal dose, as well as an individual's capacity for carcinogen bioactivation (Skipper et al., 1994). Thus, measuring protein adducts may provide an indication of the potential risk of cancer following exposure.

PhIP has been shown to bind to the blood protein albumin in humans and the binding is relatively stable (Dingley et al., 1999). However, although protein adducts of PhIP are likely to provide an important biomarker of individual exposure and metabolic capacity, there has been very little and incomplete PhIP-adduct characterization. A protein adduct of the HCA 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) has been fully characterized and forms a cysteine adduct with albumin via a sulfinamide linkage (Turesky et al., 1987). An

Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; HCA, heterocyclic amine; LC–ESI-MS, liquid chromatography–electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; HPLC, high performance liquid chromatography; m/z, mass-to-charge ratio

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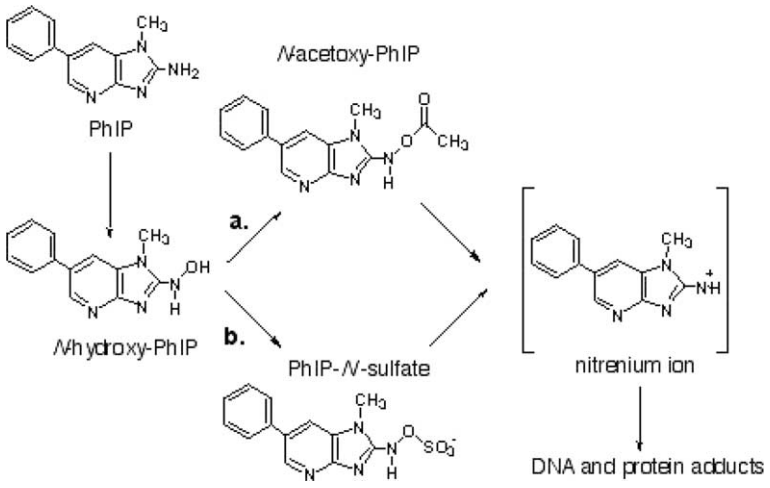

Fig. 1. Proposed metabolic activation of PhIP, where (a) and (b) correspond to acetyltransferase and sulfotransferase activities, respectively (Buonarati et al., 1990; Boobis et al., 1994).

analogous adduct was not identified from in vitro reactions between 2-nitro-PhIP and rat serum albumin. This derivative formed a product in which the 2-amino group of PhIP was lost and PhIP was linked to cysteine through a C–S bond. However reactions between *N*-acetoxy-PhIP and cysteine, or GSH resulted in unstable conjugates, which although not fully characterised, based on mass spectral analysis are consistent with structures of RS(–S–)–(H)N2–PhIP (Reistad et al., 1994).

Our aim was to identify and more fully characterize a protein adduct formed by PhIP, which could be used as a biomarker of PhIP exposure and bioactivation. This will be of great use in molecular epidemiology studies aimed at understanding the role of PhIP in cancer development. Towards this goal, we have characterized a peptide adduct formed by the putative genotoxic metabolite, *N*-acetoxy-PhIP. Previous results from our laboratory have shown that PhIP binds to albumin in the blood of humans (Dingley et al., 1999), however the large amount of non-adducted protein and subsequent digested peptides would possibly hinder the initial characterization of these protein adducts by traditional mass spectrometry techniques without prior enrichment. Therefore, a model system containing a peptide with one each of the 20 common amino acids and an internal sequence identical to rat and homologous to human albumin, *Leu–Gln–Lys–Cys–Pro–Tyr*, was chosen for the initial analysis. This homologous sequence also represents the only cysteine in human and rat albumin that is not involved in disulfide bridges within the protein, and its slightly exposed nature and nucleophilic character make it a likely candidate for protein binding (Sugio et al., 1999). Additionally, previous results have shown the formation of protein adducts via cysteine from exposure to IQ and PhIP (Turesky et al., 1987; Reistad et al., 1994).

2. Materials and methods

Materials. The peptide VSATWHLQKCPYERMDFNIG was synthesized and purified commercially (SynPep Corporation, Dublin CA). *N*-acetoxy-PhIP was synthesized and purified as described previously (Brown et al., 2001). HPLC grade solvents and all other chemicals were purchased from Aldrich (St. Louis, MO).

Covalent adduct formation. Two hundred micrograms of the model peptide was reacted with an equimolar amount of *N*-acetoxy-PhIP in potassium phosphate buffer, pH 7, with a total reaction volume of 1 ml. *N*-acetoxy-PhIP was dissolved in methanol just prior to use and was added to the reaction in 3 aliquots over 60 min. The reaction was stirred at 37 °C for 60 min and then the sample was dried and redissolved in 100 μl 10% acetonitrile containing 0.1% formic acid. The reactions with subsequent LC–MS/MS analyses were repeated in triplicate to ensure reproducibility of the results. LC–MS/MS was also repeated after storing the reaction for 5 days at room temperature.

HPLC methods. A microbore HPLC unit equipped with an autoinjector (Shimadzu Scientific, Inc., Columbia, MD) was used as the inlet for the mass spectrometer. Without prior cleanup, 5 μl of the sample was loaded onto a 1.0 mm ID × 250 mm C_{18} column (Vydac 238MS51, Hesperia, CA) equilibrated in water with 0.1% formic acid (Solvent A) and 15% acetonitrile with 0.08% formic acid (Solvent B) at 50 μl/min. The mixture was eluted using a gradient of 40% B in 50 min.

Mass spectrometry. An LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) operating in the positive mode was used. The ionization source was operated under normal spray conditions with total flow rates of 50 μl/min and a sheath gas flow of 60 psi. Typical conditions included a spray voltage of approx-

imately 5 kV and a capillary temperature of 250–270 °C. Specific focus parameters included 21 V to the heating capillary and –20 V to the tube lens. ESI(+)MS/MS was carried out using the following conditions: isolation width was 1.2 m/z, activation amplitude was 28%. Activation time was 60 ms, and the activation Q was 0.27. For third-stage MS experiments, the activation amplitude was 32% and all other parameters remained the same. Xcalibur software was used to control the mass spectrometer and the HPLC as well as analyze the data. Protein Prospector Internet-based software (UCSF) was used to predict ion fragmentation.

3. Results and discussion

The covalent species formed during the reaction of *N*-acetoxy-PhIP and a model peptide in vitro was identified using microbore HPLC coupled to an LCQ ion trap mass spectrometer. The control reactions (peptide alone and *N*-acetoxy-PhIP alone) were also monitored using LC–MS (Fig. 2). The results of the control experiments illustrate a single peak in the base peak chromatogram for the peptide and an unresolved broad peak in the base peak chromatogram for the *N*-acetoxy-PhIP control. The spectrum revealed that the starting material without the presence of a biomolecule quickly converts into *N*-hydroxy-PhIP; the expected m/z 283.1 of *N*-acetoxy-PhIP could not be extracted from the chromatogram. Conversely, the peptide is quite stable in the reaction conditions. The spectrum revealed that the peak contained a majority of the 20-mer peptide, m/z 1198.5, and a small amount of degradation product that was identified as the peptide with a loss of 2 amino acids and water at the C-terminus. In the reaction of *N*-acetoxy-PhIP and the model peptide, the chromatogram revealed an adduct species with a m/z 1309.5, corresponding to the doubly charged ion of the peptide mass with a covalent modification by PhIP, indicated by an increase in mass by 222.0 Da (Fig. 3). Importantly, the adduct was still present after storage at 25 °C for 5 days. This apparent stability is in agreement with previous work in

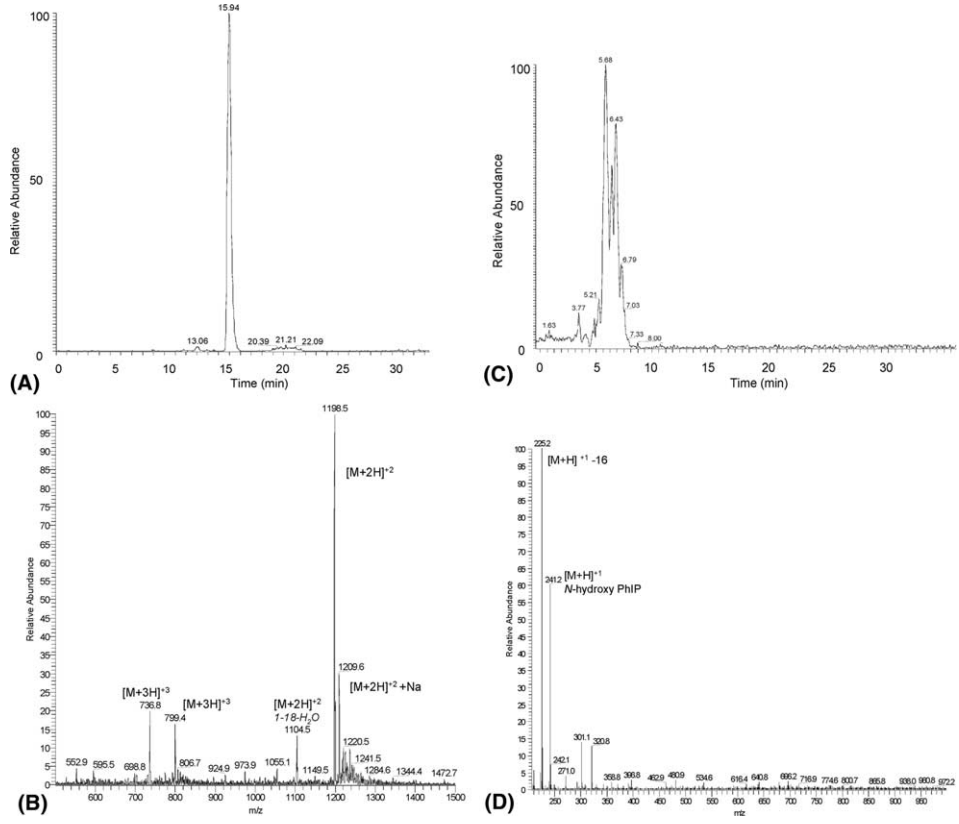

Fig. 2. (A) Base peak chromatogram of the peptide VSATWHLQKCPYERMDFNIG in a control reaction. (B) The average spectrum corresponding to the peak at 15.9 min shown in panel A. The peptide was identified by m/z 1198.5. (C) Base peak chromatogram of *N*-acetoxy-PhIP control reaction. (D) The average spectrum corresponding to the peaks between 5 and 7 min shown in panel C. The degradation of *N*-acetoxy-PhIP to *N*-hydroxy-PhIP was confirmed by the presence of the singly charged ion, m/z 241.2.

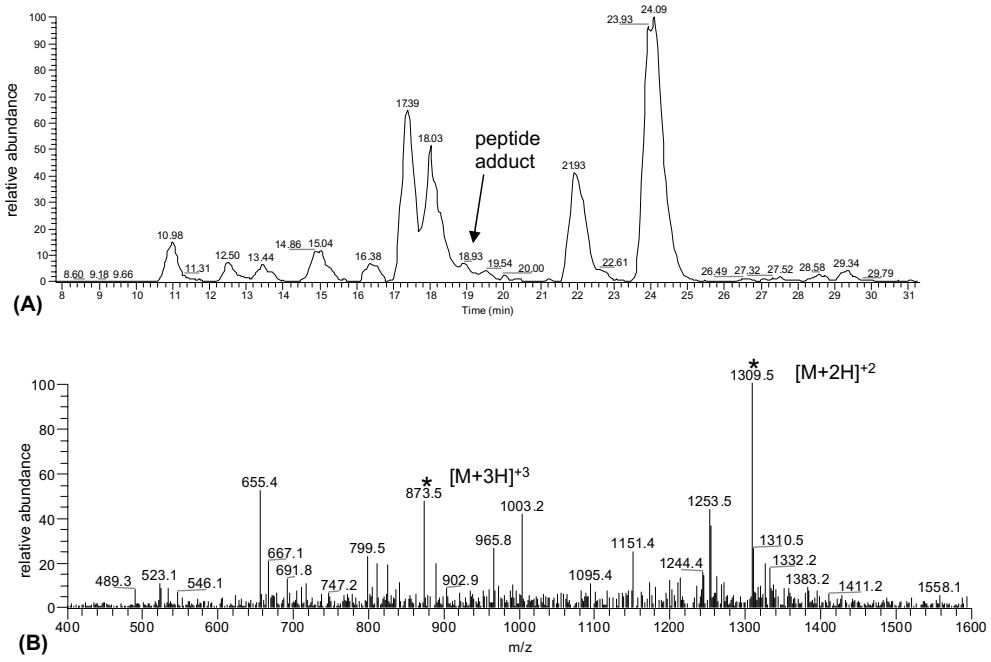

Fig. 3. (A) Base peak chromatogram of peptide-PhIP reaction. The arrow (retention time: 18.9 min) indicates the elution of the peptide-PhIP adduct. (B) The average spectrum corresponding to the peak at 18.9 min shown in panel A. The adduct was identified by the presence of doubly and triply charged ions, $m/z = 1309.5$ and 873.5, respectively.

our laboratory that showed the formation of stable adducts in vivo measured in human plasma after the administration of PhIP (Dingley et al., 1999).

As illustrated in Fig. 3, there are a number of unidentified peaks in the base peak chromatogram. Even though the chromatogram indicates that the covalent species is a minor product, it was possible to manually extract 1309.5 as a predicted adduct species from the chromatogram; this species tailed significantly after the elution of the unreacted peptide. However, the other peaks did not correspond to any predicted side products in the reaction such as additional covalent adducts to the peptide or *N*-acetoxy-PhIP by-products. It was predicted that the *N*-acetoxy-PhIP by-products would be prominent in the reaction monitoring, so we scanned a range from 400 to 1600 m/z. Indeed, the peak at 1309.5 was the only peak we could discern resulting from the reaction of *N*-acetoxy-PhIP and the peptide. Other predicted molecular weights of adducts could not be extracted from the chromatogram. Presumably the unidentified peaks are derived from the high molecular weight peptide, as the protonated PhIP $[M+H]^+$ at m/z 225 and by-products would be below mass m/z 500.

The initial MS analysis and the molecular weight of the adduct species indicates a nucleophile displacing the acetyl group and possibly suggests a linkage via the exocyclic amino group of *N*-acetoxy-PhIP. Relative to the peak intensity of the unreacted peptide in the mixture, it is predicted that roughly ~15% of the adduct was formed. This is an approximate value since there wasn't a calibration curve constructed for the unreacted peptide.

The adduct species was further characterized by LC–ESI(+)-MS/MS to determine the exact amino acid containing the modification. Although hypothesized to be cysteine, other amino acids such as lysine or arginine could form adducts with electrophilic metabolites like the HCAs, especially if they are exposed in a peptide with little or none secondary structure. The doubly-charged species, $[1309.5]^{+2}$, was selected for fragmentation in an LC–MS/MS experiment, and the recorded spectrum is shown in Fig. 4. The fragment ions generated, mostly singly charged *b*- and *y*-ions (Biemann, 1990), were consistent with the predicted fragmentation pattern for the 20-amino acid peptide. Other ions, $y_{11} - y_{14}$, $b_{12} - b_{13}$, and internal fragment 7–12, were present and correspond to the PhIP modification observed from the parent peptide adduct mass. The cysteine was therefore determined to be the site of covalent modification by deduction from the unmodified and modified fragment ions (Fig. 4B). Other covalent modifications that have been reported using other metabolic forms of PhIP were not observed in these reactions (Reistad et al., 1994). Additionally, this covalent adduct is different from the sulfinamide linkage observed between serum albumin and IQ, in that the cysteinyl is not oxidized (Turesky et al., 1987).

The intensity of the modified fragment, y^*_{11}, m/z 1566.5 made it possible to select this ion for further fragmentation to gain more structural detail. A

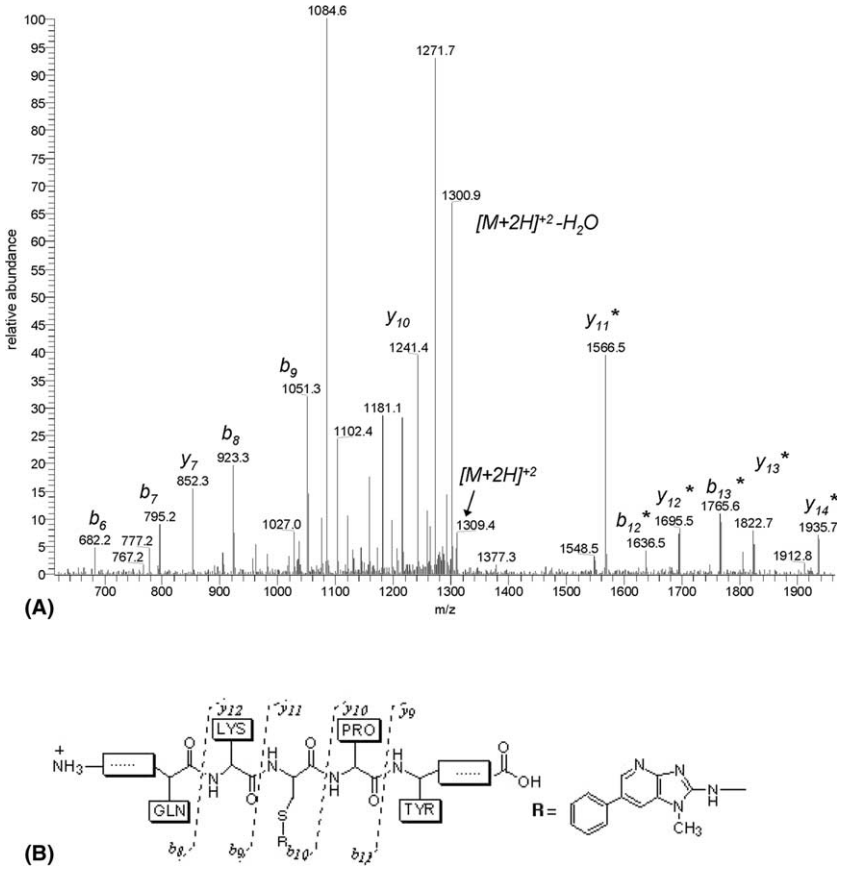

Fig. 4. (A) Spectrum from tandem MS experiment of m/z 1309.5. The sequence specific b- and y-ions and internal fragment ions are assigned above the corresponding peak. Fragment ions with an increased mass of +222.0, the corresponding change that would be represented by a covalent attachment of PhIP, are marked with an asterisk. The spectrum was recorded using an activation amplitude of 28% in the ion trap detector. (B) Amino acid sequence and predicted fragmentation pattern surrounding the cysteinyl-PhIP adduct. The proposed structure of the PhIP-peptide adduct is shown.

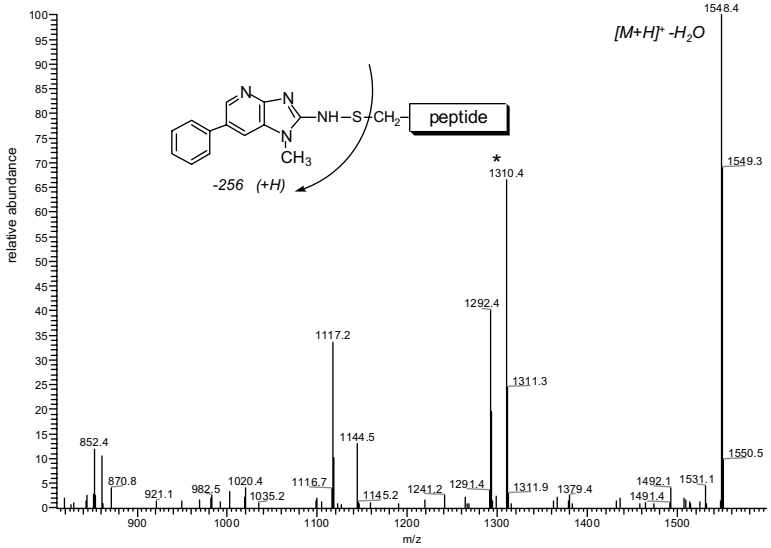

Fig. 5. Spectrum from a 3-stage MS experiment of m/z 1309.5 and the y_{11}^{*} ion fragment ion m/z 1566.5. The asterisk denotes the loss of 256 and the corresponding structure is illustrated. The spectrum was recorded using an activation amplitude of 28% and 32%, respectively, in the ion trap.

three-stage MS experiment was performed with the same parameters described for the MS/MS of m/z 1309.5 with subsequent fragmentation of m/z 1566.5 (Fig. 5). After loss of water from this daughter ion, the next most abundant ion in the spectrum was a loss of 256 m/z 1310.4. This loss may suggest a structure with the amino-sulfur linkage intact, and this type of fragmentation pattern is seen with MS/MS experiments of the reaction products of PhIP and cysteine (data not shown). This further supports the structure of the covalent modification shown in Fig. 4. Ultimately the structure we have suggested will require confirmation by ^{1}H-NMR.

This is the first MS/MS characterization of a covalent adduct formed between a peptide or protein and *N*-acetoxy-PhIP. It is proposed that the results herein may be consistent with adduct formation in vivo since there is a free cysteine in rat and human serum albumin that is suitably poised for interaction with the HCA. This does not preclude, however, that this is the only adduct formed in vivo and studies are underway to test these hypotheses.

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