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Optimized enzymatic hydrolysis of DNA for LC–MS/MS analyses of adducts of 1-methoxy-3-indolylmethyl glucosinolate and methyleugenol

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ABSTRACT

Mass spectrometric analyses of DNA adducts usually require enzymatic digestion of the DNA to nucleosides. The digestive enzymes used in our laboratory included a calf spleen phosphodiesterase, whose marketing was stopped recently. Using DNA adducted with bioactivated methyleugenol and 1-methoxy-3-indolylmethyl glucosinolate—each forming dA and dG adducts—we demonstrate that replacement of calf spleen phosphodiesterase (Merck) with bovine spleen phosphodiesterase (Sigma–Aldrich) leads to unchanged results. Enzyme levels used for DNA digestion are extremely variable in different studies. Therefore, we sequentially varied the level of each of the three enzymes used. All dose (enzyme)–response (adduct level) curves involved a long plateau starting below the enzyme levels employed previously. Thus, we could reduce the amounts of micrococcal nuclease, phosphodiesterase, and alkaline phosphatase for quantitative DNA digestion by factors of 4, 2, and 333, respectively, compared to our previous protocols. Moreover, we observed significant phosphatase activity of both phosphodiesterase preparations used, which may affect the recovery of adducts with methods requiring digestion to 2′-deoxynucleoside-3′-monophosphates (e.g., ^{32}P-postlabeling). In addition, the phosphodiesterase from Sigma–Aldrich, but not that from Merck, deaminated dA. This was irrelevant for the dA adducts studied, involving bonding at N^6, but might complicate the analysis of other dA adducts.

DNA adducts represent a major primary damage that can lead to the induction of gene mutations. Usually, DNA adducts are detected using radiolabeled test compounds, postlabeling of adducts (e.g., with ^{32}P), immunochemistry, and capillary electrophoretic or chromatographic techniques coupled with fluorescence or mass spectrometric detection [1–3]. With only few exceptions (e.g., immunohistochemistry), these techniques require enzymatic hydrolysis of the DNA to nucleosides or nucleotides prior to analysis. Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)[2] has substantially gained in sensitivity during the past decade and is now frequently used for the determination of DNA adducts [1]. Thereby, the analysis of adducted 2′-deoxynucleosides by multiple-reaction monitoring (MRM) offers the highest sensitivity for the detection of the majority of DNA adducts [1,4]. For quantification of DNA adducts after digestion to 2′-deoxynucleosides, it is important to ensure complete digestion of the DNA.

Recently, we developed LC–MS/MS-based methods for the analysis of DNA adducts formed by some food-borne genotoxicants, such as alkyl-substituted polycyclic hydrocarbons [5], hydroxymethyl-substituted furans [6,7], and certain secondary plant metabolites [8,9]. Previously, we used the ^{32}P-postlabeling technique for detecting some of these DNA adducts [5,10]. Standard protocols are available for the ^{32}P-postlabeling assay [2,11]. They involve the digestion of DNA to 2′-deoxynucleoside-3′-monophosphates by micrococcal nuclease and calf spleen type II phosphodiesterase prior to the labeling step. Generally, phosphodiesterase produced by Merck (Germany) was used. We kept this digestion procedure for the mass spectrometric analyses, but added a further digestion step, the conversion of the nucleotides to nucleosides in the presence of alkaline phosphatase.

Recently, Merck stopped the marketing of its calf spleen type II phosphodiesterase. Therefore, we had to find an alternative. Moreover, larger DNA samples have to be digested and much larger numbers of samples can be handled in LC–MS/MS-based assays than with the ^{32}P-postlabeling technique. These factors led to dramatic increases in our costs for enzymes. Moreover, a literature search indicated that digestion protocols differ drastically between various laboratories that use LC–MS/MS for DNA adduct analyses (Table 1). Therefore, we decided to optimize the digestion conditions. We used DNAs modified by two different genotoxicants for this optimization. Both genotoxicants, 1-methoxy-3-indolylmethyl

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[2] *Abbreviations used:* dA, 2′-deoxyadenosine; 3′-dAMP, 2′-deoxyadenosine-3′-monophosphate; dG, 2′-deoxyguanosine; 3′-dGMP, 2′-deoxyguanosine-3′-monophosphate; dI, 2′-deoxyinosine; ESI, electrospray ionization; LC, liquid chromatography; MIE, *trans*-methylisoeugenol-3′-yl; 1-MIM, 1-methoxy-3-indolylmethyl; MRM, multiple-reaction monitoring; MS/MS, tandem mass spectrometry; UPLC, ultra-performance liquid chromatography.

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Table 1
Kinds and levels of enzymes used for the digestion of DNA for LC–MS/MS-based DNA adduct analyses in various laboratories. [a]

Study	Adducting agent	Enzyme amount, mU/μg DNA				
		Phosphodiesterase type II	Phosphodiesterase type I	Micrococcal nuclease (*S. aureus*)	Nuclease P1	Alkaline phosphatase
Herrmann et al. [8]	Methyleugenol	4.2[b]		213		240[c]
Schumacher et al. [9]	1-MIM glucosinolate	4.2[b]		213		160[c]
Neale et al. [19]	Aromatic amines	0.07–0.12[d]		33–59		33–59[e]
Paini et al. [20]	Estragole	0.1[d]	0.07[f]		0.1[g]	76[c]
Delatour et al. [21]	Oxchratoxin A	0.24[d]	0.05[f]		0.1[g]	40[c]
Lao et al. [22]	Tobacco-specific nitrosamines	0.5–0.9[d]		75–150		150–300[c]
Monien et al. [6,7]	5-Hydroxymethylfurfural, furfuryl alcohol	0.53[b]		26		30[h]
Doerge et al. [23]	Chloroacetaldehyde	2.0[d]		20	0.05[g]	
Zhang et al. [24]	Acrolein	2.3–9[d]		375–1500		750–3000[c]
Matsuda et al. [25]	Ethanol	3.75[i]		1125		150[e]
Chen et al. [26]	Acrolein, croton aldehyde	4.5[d]		625		1500[c]
Chen et al. [27] [j]	Lipid peroxidation products	10[d]		100		20[e]
Regal et al. [28]	3-Methylindole	1333[b]		333	444	
This study, recommended protocol	Methyleugenol, 1-MIM glucosinolate	2.1[d]		53		0.6[c]

[a] We listed only protocols involving the usage of phosphodiesterase type II. This enzyme is mostly used in combination with micrococcal nuclease, as both enzymes generate 3′-phospho-containing products. The major alternative involves the application of DNase I in combination with phosphodiesterase type I, enzymes forming 5′-phosphorylated degradation products (e.g., [29–31]).
[b] Calf spleen.
[c] Calf intestine.
[d] Bovine spleen.
[e] Source not specified.
[f] *Crotalus adamanteus.*
[g] Micrograms of enzyme protein per microgram of DNA.
[h] Shrimp.
[i] Spleen, not further specified.
[j] Additional usage of 30 mU/μg DNA adenosine deaminase.

(1-MIM) glucosinolate and methyleugenol, are secondary plant metabolites. Both form dA as well as dG adducts. The structures of these test compounds and the resulting adducts as well as the activation pathways involved are displayed in Fig. 1. 1-MIM glucosinolate is present at high levels (up to 80 μg/g fresh wt) in various *Brassica* vegetables, such as broccoli, cauliflower, and cabbage [12]. Methyleugenol is a carcinogen found in fennel and many herbal spices, such as basil, anise, and tarragon [13].

We utilized three enzymes for the digestion of DNA: (i) micrococcal nuclease has exo- and endonucleolytic activity toward DNA and RNA, generating 3′-phosphomononucleosides and -oligonucleotides; (ii) type II phosphodiesterases (from calf or adult bovine spleen) are exonucleases that generate 3′-phosphomononucleosides from 5′-hydroxy-terminated ribo- and deoxyribo-oligonucleotides; and (iii) alkaline phosphatase from calf intestine nonspecifically cleaves off phosphate groups from numerous phosphate monoesters, including nucleotides. It should be added that the enzymes used were purified from biological sources. They may show side activities, intrinsically or due to incomplete removal of other enzymes [14]. Moreover, the presence of adducts might affect the rate of hydrolysis of nucleic acids. Therefore, we used DNAs with high and low adduct levels in our investigation.

Materials and methods

Chemicals and enzymes

Methyleugenol, 2′-deoxyinosine (dI), herring sperm DNA, micrococcal nuclease from *Staphylococcus aureus*, phosphodiesterase (type II) from bovine spleen, alkaline phosphatase from calf intestine, and myrosinase from *Sinapis alba L.* seed were purchased from Sigma–Aldrich (Taufkirchen, Germany). Phosphodiesterase (type II) from calf spleen was obtained from Merck (Darmstadt, Germany). 3′-dAMP and 3′-dGMP were from Carbosynth (Compton, UK). [$^{15}N_5$]dG and [$^{15}N_5$]dA were purchased from Silantes (Munich, Germany). 1-MIM glucosinolate was isolated from lyophilized broccoli [10] and 1-MIM alcohol was synthesized as reported previously [15]. The synthesis of isotope-labeled adducts was described in [9] for [$^{15}N_5$]N^2-(1-MIM)-dG and [$^{15}N_5$]N^6-(1-MIM)-dA and in [8] for [$^{15}N_5$]N^2-MIE-dG and [$^{15}N_5$]N^6-MIE-dA.

Formation of DNA adducts and isolation of DNA

Modification of DNA with both test compounds was conducted in vitro as well as in a mouse model in vivo. For formation of 1-MIM adducts in vitro, herring sperm DNA (2 mg) was incubated with 1-MIM glucosinolate (10 nmol) and myrosinase (100 mU) in 1 ml of 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 20 h. For formation of MIE adducts in vitro, herring sperm DNA (20 mg) was incubated with 1′-acetoxymethyleugenol (100 nmol) in 10 ml water at 37 °C for 1 h. The adducting agents and their decomposition products were removed as described previously [8,9], and the DNA was precipitated with ethoxyethanol or isopropanol and NaCl, washed with ice-cold 70% ethanol in water, and then dissolved in water [8,9].

An 8-week old male FVB/N mouse from Harlan (Rossdorf, Germany) and a 10-week old male transgenic mouse carrying the human *SULT1A1/1A2* gene cluster [16] were treated once with either 600 μmol 1-MIM alcohol or 281 μmol methyleugenol per kilogram body mass by gavage using tricaprylin as vehicle. The 1-MIM alcohol-treated mouse and the methyleugenol-treated mouse were sacrificed 8 and 24 h, respectively, after treatment. DNA was isolated from homogenized hepatic tissue by standard phenol–chloroform extraction [17].

Digestion of DNA

Aliquots of 12.5 or 25 μg DNA (for studying MIE and MIM adducts, respectively) were dried under reduced pressure and then