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Isothiocyanates may chemically detoxify mutagenic amines formed in heat processed meat

Anna Lewandowska [a,c], Witold Przychodzeń [b], Barbara Kusznierewicz [c], Dominik Kołodziejski [c], Jacek Namieśnik [a], Agnieszka Bartoszek [c]

[a] Department of Analytical Chemistry, Chemical Faculty, Gdansk University of Technology, Poland, 11/12 Narutowicza Str., 80-233 Gdansk, Poland
[b] Department of Organic Chemistry, Chemical Faculty, Gdansk University of Technology, Poland, 11/12 Narutowicza Str., 80-233 Gdansk, Poland
[c] Department of Food Chemistry, Technology and Biotechnology, Chemical Faculty, Gdansk University of Technology, 11/12 Narutowicza Str., 80-233 Gdansk, Poland

a b s t r a c t

Meat consumption represents a dietary risk factor increasing the incidence of common cancers, probably due to carcinogenic amines (HAAs) formed upon meat heating. Interestingly, cancers whose incidence is increased by meat consumption, are decreased in populations consuming brassica vegetables regularly. This inverse correlation is attributed to brassica anticarcinogenic components, especially isothiocyanates (ITCs) that stimulate detoxification of food carcinogens. However, ITC reactivity towards amines generating stable thioureas, may also decrease mutagenicity of processed meat. We confirmed here that combining meat with cabbage (fresh or lyophilized), in proportions found in culinary recipes, limited by 17–20% formation of HAAs and significantly lowered mutagenic activity of fried burgers. Moreover, MeIQx mutagenicity was lowered in the presence of ITCs, as well as for synthetic ITC-MeIQx conjugates. This suggests that formation of thioureas could lead to chemical detoxification of food carcinogens, reducing the cancer risk associated with meat consumption.

Keywords: Pork burger, Heterocyclic aromatic amines, Isothiocyanates, Chemical detoxification

1. Introduction

Diet is a major risk factor in human cancer and meat is the main dietary ingredient behind this increased incidence of carcinogenesis. This observation was brought to public attention for the first time by the results of classic analysis by Doll and Peto (1981) and has been confirmed by a number of meta-analyses (Genkinger & Koushik, 2007; WCRF/AICR, 2007; Parkin, 2011) and also by recently published results of network case–control studies (Di Maso et al., 2013). Although initially disputable, the growing body of evidence points to the association between the intake of heterocyclic aromatic amines (HAAs) formed upon heat processing of meat and the risk of common cancers, such as colorectal (Fu et al., 2011), bladder (Lin et al., 2012), prostate (Major et al., 2011), breast, colon, pancreatic (Zheng & Lee, 2009) or lung (Lam, Cross et al., 2009; Lam, Gallicchio et al., 2009). These compounds are formed during heat processing in ng per g of meat amounts, but are highly mutagenic, and their carcinogenicity is dozens of times higher than that of other genotoxic food carcinogens, such as aflatoxin B_1 or nitrosoamines, and also much higher than that of benzo[α]pyrene (Püssa, 2013). Interestingly, consumption of brassica vegetables (cabbage, broccoli and cauliflower) decreases the incidence of the same types of cancers – colorectal (Wu et al., 2013), prostate (Kristal & Lampe, 2002), breast (Terry, Wolk, Persson, & Magnusson, 2001), bladder, colon, pancreatic (Bosetti et al., 2012) and lung (Lam, Cross et al., 2009; Lam, Gallicchio et al., 2009).

The most important bioactive phytochemicals produced by brassica plants are glucosinolates, that are degraded by the endogenous enzyme myrosinase and modifier proteins to a number of products, among which isothiocyanates (ITCs) exhibit the strongest anticarcinogenic potential (Dinkova-Kostova & Kostov, 2012). These compounds trigger an array of cytoprotective mechanisms and were shown to affect HAA metabolism (Murray et al., 2001; Walters, 2004) and detoxification (Steck & Hebert, 2009) in humans, that may diminish the carcinogenic effects of processed meats. However, the ability to stimulate human organism's own defense against xenobiotic insult is not the only possible way

Abbreviations: AITC, allyl isothiocyanate; HAA, heterocyclic aromatic amine; ITC, isothiocyanate; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; PEITC, phenethyl isothiocyanate; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; SFN, sulforaphane.

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attributable to ITCs by which they may reduce cancer risk associated with thermally processed meat consumption. Chemical properties of ITCs make them conducive to the reaction with amines. Such a reaction with the amino group of HAAs leading to the formation of thioureas could prevent the formation of genotoxic N^2-hydroxy derivatives. Since heat processed meat is often either combined during preparation or served with brassica vegetables, there is possibility for such chemical processes to occur before food absorption from alimentary tract takes place. In this study, we tested whether the proposed above *ex vivo* chemical detoxification of carcinogenic food amines could diminish their mutagenic potential.

2. Materials and methods

2.1. Preparation of pork burgers

Raw pork (22% fat) lard and French bread loaf were purchased in a local supermarket (Poland). White cabbage (*Brassica oleracea* var. *capitata* f. *alba*), Kamienna Glowa variety, originated from a nearby organic farm and contained about 10 μmoles of glucosinolates per g of dried weight. From the bread loaf, the crust was removed and the flesh was dried then grated. The meat was minced and manually mixed with either 20% [w/w] of minced frozen cabbage or 4% [w/w] of the same cabbage lyophilisate or 4% of dry breadcrumbs. To the two latter samples, 16% [v/w] of water was added to maintain the proportion 20 g of additives per 100 g of meat mixture. The amount of water in the frozen cabbage was established based on the 80% weight loss upon cabbage lyophilisation. The 60 g portions of meat mixtures were formed into burgers and fried in lard for 5 min on each side, which ensured that they become very well-done. Fried burgers were stored in freezer bags at −18 °C until extraction of HAAs.

2.2. Extraction of MeIQx and PhIP from the outer and inner layers of burger samples

The HAAs were extracted and purified using a solid-phase extraction method adopted from Gross and Grüter (1992) with some modifications. One extract was prepared from one burger. Fried meat (6 g), outer and inner layers separately, was homogenised in 18 ml of 1 M NaOH, mixed with 20 g of diatomaceous earth and transferred into blank Extrelut NT 20 columns (Merck, Germany). The amines were extracted with 80 ml of eluent consisting of dichloromethane:toluene 95:5 (v/v) directly to propanesulfonic acid (PRS) cationic exchanger cartridges (J.T. Baker, Germany), containing 0.5 g of sorbent preconditioned with 4 ml of the eluent. The sorbent was dried and rinsed with the following sequence of eluents: 6 ml of 0.1 M HCl, 15 ml of MeOH:0.1 M HCl 6:4 (v/v) and 2 ml of water. In this way, less polar compounds (e.g. PhIP) were eluted. The combined acidic solutions were mixed with 25 ml of water, then neutralised with 0.5 ml of aqueous ammonia (25% v/v). These less polar HAAs were subsequently adsorbed on C_{18} (0.5 g) cartridges (J.T. Baker, Germany) preconditioned with a mixture of 5 ml methanol and 5 ml water. This fraction of amines was eluted with 2 ml of mixture composed of methanol:25% aqueous ammonia 9:1 (v/v). To recover the more polar HAAs (e.g. MeIQx and residues of PhIP), the PRS cartridges were coupled with octadecylsilane C_{18} (0.1 g of sorbent) cartridges (J.T. Baker, Germany) preconditioned with a mixture of 5 ml methanol and 5 ml water. The adsorbed polar HAAs were eluted with 25 ml of 0.5 M ammonium acetate, pH 8.5. After washing C_{18} cartridges with ultrapure water (5 ml) and drying, the polar fraction of amines was recovered from the C_{18} cartridges with 2 ml of a mixture composed of methanol:25% aqueous ammonia 9:1 (v/v). Both fractions of HAAs, after drying in nitrogen flow in an evaporator, were redissolved in 0.5 ml of MeOH each and 0.25 ml samples were submitted to chromatographic analysis. The remaining portions of these solutions were again dried in nitrogen flow in an evaporator, dissolved in 0.25 ml of DMSO and subjected to Ames assay.

2.3. Chromatographic analysis of HAAs in burger extracts

The content of MeIQx and PhIP in the extracts from fried burgers was assessed by the modified method described by Gorlewska-Roberts, Teitel, Lay, Roberts, and Kadlubar (2004). An Agilent Technologies 1200 Series HPLC–DAD system connected to API–ESI–MS Agilent 6130 Quadrupole LC/MS (*Agilent Technologies*, USA) was used throughout the study. Chromatographic separations were performed on an *Agilent Zorbax RX-*C_{18} column (150 × 4.6 mm, 5 μm). The mobile phase was a mixture of water with 0.01% formic acid (A) and 95% acetonitryle with 0.01% formic acid (B). The HPLC program was 100% A for 4 min followed by a linear gradient to 10% A till 30 min and 5 min post-run delay. The flow rate was set at 0.7 ml/min and the injection volume was 50 μl. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 180 V; drying gas temperature, 350 °C; gas flow (N_2), 12 l/min; nebulizer pressure, 35 psig. The instrument was operated in positive ion mode and selected ion monitoring (SIM) was used to detect m/z 214.1 (for MeIQx), and m/z 225.1 (for PhIP). Instrument control data acquisition and data analyses were carried out with ChemStation (*Agilent Technologies*, USA). Calibration standards were prepared by diluting the stock solutions of MeIQx and PhIP (*Toronto Research Chemicals Inc.*, Canada) with methanol in a range from 0.1 to 5 ng/μl and from 0.01 to 0.5 ng/μl, respectively. Calibration curves were obtained by injecting 1 μl of standard solutions to the HPLC–DAD–MS analysis.

2.4. Mutation assay using microplate Ames test (MPF)

Mutagenicity assessments were carried out for MeIQx, reaction mixtures consisting of MeIQx and ITCs (allyl isothiocyanate, AITC, or phenethyl isothiocyanate, PEITC, both from *Sigma–Aldrich,* UK, and sulforaphane, SFN, synthesised at the Gdansk University of Technology, Poland), corresponding synthetic ITC-MeIQx thioureas (synthesis and characterisation presented in Supplementary), as well as the extracts of HAAs obtained from outer and inner layers of pan-fried burgers. The induction of mutations was examined by a microplate version of the Ames assay (*Xenometrix*, Switzerland) using two tester strains: *Salmonella typhimurium* TA98 (*Xenometrix*, Switzerland) or *S. typhimurium* YG1024 overexpressing N-hydroxylamine O-acetyltransferase (OAT) kindly provided by Dr. T. Nohmi from the National Institute of Health Sciences, Japan. All tests were carried out without and with metabolic activation by Aroclor-induced rat liver microsomal fraction S9 (*Xenometrix*, Switzerland). The methodology followed strictly that recommended by the producer (http://www.xenometrix.ch/index.php?id=61).

2.5. Stability of thioureas in the presence of S9-mix

In order to investigate the stability of three studied ITC-MeIQx thioureas under Ames test conditions, a 0.64 ml portion of thiourea solution (final concentration in the mixture 2 μM) was mixed with the growth (6 ml) and exposure (0.8 ml) media, as well as 0.6 ml of S9-mix (S9 fraction with cofactors). The mixtures were stirred at 37 °C and the samples (0.4 ml) were collected every 30 min for 7 h and once after 24 h. To precipitate proteins, the collected samples were immediately mixed with 0.4 ml of mixture consisting of phenol:chloroform:isoamyl alcohol (25:24:1) supplemented with 1 mM EDTA and buffered with 10 mM Tris, pH 8. The mixtures

were mixed vigorously and then centrifuged at 4000 *g* for 4 min to separate layers. The upper layer was discarded, while 0.3 ml aliquot of the bottom layer was transferred into the analysis vial. The content of the appropriate thiourea and MeIQx in each sample were determined by LC–DAD–ESI–MS in the same run (chromatographic conditions as described for HAA analysis). The instrument was operated in a positive ion mode and to quantify degradation rate of thioureas, the data were acquired in SIM mode for MeIQx (*m*/*z* 214.1) or respectively for AITC-MeIQx (*m*/*z* 313.1), PEITC-MeIQx (*m*/*z* 377.0), and SFN-MeIQx (*m*/*z* 391.5).

2.6. Statistical analysis

All statistical analyses were performed using the Prism 4.0 software package (GraphPad Software, Inc.) applying either *t*-Student test or ANOVA with Dunnet's test or Pearson's correlation. The level of statistical significance was set at $P \leqslant 0.05$.

3. Results and discussion

3.1. Determination of HAA in pan-fired burgers

A number of dietary factors have been shown to impact the content of HAAs in heat treated meat foods (Alaejos & Afonso, 2011), however the influence of the addition of brassica vegetables has not been studied despite such a culinary tradition in many local cuisines. Therefore, in the first step, we measured how cabbage phytochemicals influence the formation of the most prominent HAAs – 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (Bartoszek, 2007), as well as the mutagenic activity of pan-fried burgers. The burgers were prepared with pork mince and either minced fresh or lyophilised white cabbage; control burgers contained the corresponding amount of dry breadcrumbs. The burgers were fried using lard until very well-done, which generally yields higher levels of HAAs than other cooking methods (Ferguson, 2010), because the meat is in direct contact with a hot flat surface. The effect of addition of cabbage, on the formation of HAAs in pork burgers, was investigated separately in the outer and inner layers (Fig. 1). Representative chromatograms from the HPLC analysis obtained for the meat samples with or without cabbage are shown in Supplementary (Fig. S6). Compared to control burgers containing breadcrumbs, both fresh and lyophilized cabbage added to ground pork before frying led to the reduction of HAAs content by about 17% and 20% in the outer layers of burgers and by 4% and 9% in the inner layers of burgers, respectively (numerical data are provided in Supplementary, Table S2). The levels of PhIP were significantly reduced in both the outer and the inner layers of hamburgers after cabbage addition. There are significant differences in MeIQx levels between outer layers of burgers containing breadcrumbs and lyophilized cabbage and no significant differences in MeIQx levels between burgers containing breadcrumbs and fresh cabbage, however although the *P* value was higher than 0.05, but low enough to show the trend.

The content of MeIQx in burgers with breadcrumbs was similar to that reported in previous studies (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012). In contrast, the concentration of PhIP was unexpectedly low; such variations in the composition of HAAs formed during heat processing were discussed before and were ascribed to the varied sugar content in pork meats and the genotype of the pigs (Olsson, Skog, Lundstrom, & Jägerstad, 2002).

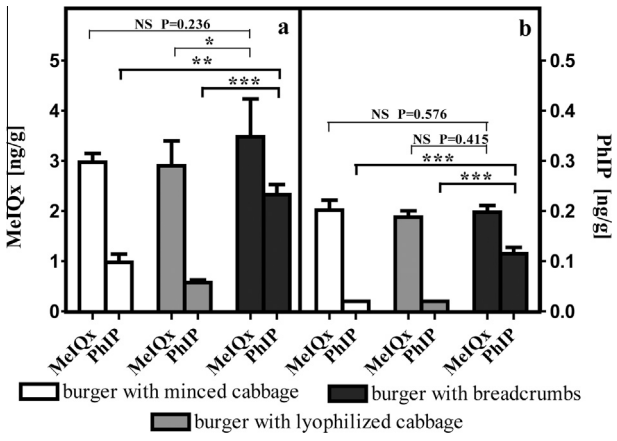

Fig. 1. The contents of MeIQx and PhIP in pan-fried burgers made from minced pork combined with either fresh or lyophilized cabbage or breadcrumbs. Concentration of HAAs was determined by LC–ESI-MS separately for the outer (a) and inner layers (b) of burger samples. The values are means ± s.e.m. of 4 independent determinations. The statistical analysis was performed by *t*-Student test: *, **, *** significantly, NS, not significantly, different from burgers with breadcrumbs, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. Determination of mutagenicity of pan-fired burgers

Apart from MeIQx and PhIP, other mutagenic components belonging to either HAAs or other groups, e.g. polycyclic aromatic hydrocarbons, can be formed during meat frying (Bartoszek, 2007). Therefore, we determined also the overall mutagenicity of prepared fried pork burgers by the Ames test. The mutagenic activity of samples was evaluated using a microplate MPF version of this test (plate photos as shown in Supplementary, Fig. S5), because of its convenience and other advantages discussed by Umbuzeiro et al. (2010). Two tester *S. typhimurium* strains were employed: TA98, a standard strain used in Ames test, and YG1024 derived from TA98 strain, but modified to overexpress N-hydroxylamine O-acetyltransferase, which makes it particularly sensitive towards HAAs (Watanabe, Ishidate, & Nohmi, 1990). The observed mutagenic activity of extracts that were used for MeIQx and PhIP content determination was significantly lower ($P < 0.01$) for outer layers of burgers with cabbage than for an outer layer of control burgers with breadcrumbs (Fig. 2). The mutagenicity of the inner layers of both cabbage burgers was not significantly different from that of the control (Fig. 2).

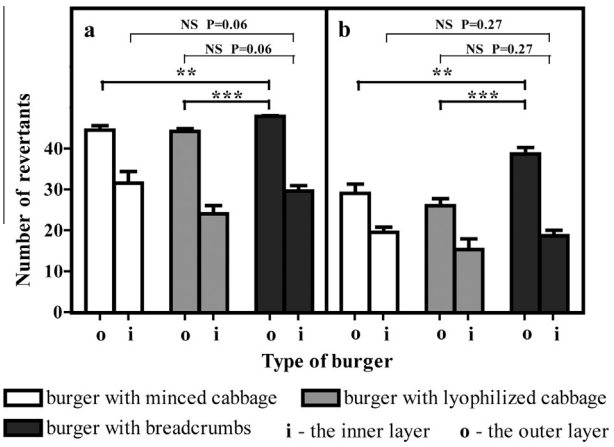

Fig. 2. Mutagenic activity of HAA extracts from outer (o) and inner layers (i) of pork burgers prepared with either fresh or lyophilized cabbage or breadcrumbs determined by a microplate version of Ames test in two *S. typhimurium* strains: (a) YG1024 overexpressing N-hydroxylamine O-acetyltransferase and (b) TA98 standard strain used in Ames test. The values are means ± s.e.m. of 6 independent determinations. The statistical analysis was performed by ANOVA with Dunnet's test: **, *** significantly, NS, not significantly, different from burgers with breadcrumbs, $^{**}P < 0.01$, $^{***}P < 0.001$.

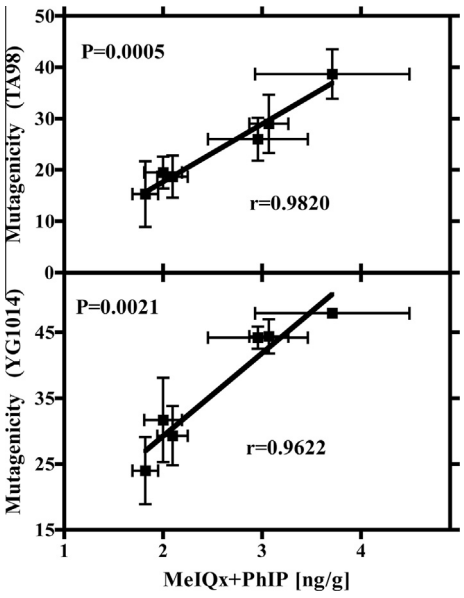

Fig. 3. The Pearson's correlation between mutagenicity of all burger samples studied determined by microplate version of Ames test (data from Fig. 2) and the sum of contents of MeIQx + PhIP measured in these samples by HPLC–ESI–MS (data from Fig. 1).

Moreover, the mutagenic response very well correlated ($r = 0.9622$, $P = 0.0021$ for YG1024 and $r = 0.9820$, $P = 0.0005$ for TA98 strains) with the sums of contents of MeIQx and PhIP determined in all fried pork burgers studied (Fig. 3), suggesting that HAAs were the major mutagens formed during pan-frying.

3.3. Determination of mutagenicity of MeIQx in the presence of ITC

The significant decrease of mutagenicity of burgers, containing cabbage, was difficult to explain solely by the lower level of HAAs, especially as the significant inhibition of their formation was observed only in the case of substantially less mutagenic PhIP. These results suggested another role for cabbage phytochemicals, in particular ITCs, which were demonstrated to form conjugates (thioureas) with aromatic amines (Śmiechowska et al., 2010).

Assuming that the reaction to thioureas could have an important role in the reduction of the mutagenicity observed for the burgers containing cabbage, we checked whether combining HAAs with ITCs would be reflected by the diminished mutagenic response in Ames test. In these experiments, HAAs were represented by MeIQx, a stronger mutagen (Jägerstad & Skog, 2005) and a possible human carcinogen (as classified by the International Agency for Research on Cancer, Lyon, France) accounting for more than 90% of the mutagenic activity found in meat moderately fried under household conditions (Sinha et al., 1998). From ITCs found in white cabbage, the three derivatives best known for anticarcinogenic properties were selected, *i.e.*, allyl isothiocyanate (AITC), phenethyl isothiocyanate (PEITC) and sulforaphane (SFN, 1-isothiocyanato-4-methylsulfinylbutane). The mutagenic activity of MeIQx, alone or combined with the mentioned ITCs, was tested by Ames test MPF using TA98 and YG1024 strains. As seen in Fig. 4, for samples where the ITCs were applied in excess (1:10 or 1:100 molar ratio), a highly significant reduction of the amine mutagenicity was shown, similar in the case of all ITCs studied. However, much lower (YG1024) or no protective (TA98) effect was observed for molar reagent ratio 1:1. Thus, under typical culinary conditions in which concentration of isothiocyanates would be expected to be in the range of μmoles/g of dish (about 4 μmoles per 1 g of burgers in this study) compared to at most nanomoles of HAAs/g meat (Oz & Kaya, 2011), the antimutagenic effect may be expected to occur.

3.4. Determination of mutagenicity of conjugates of MeIQx and ITCs

Finally, we investigated whether conjugates of MeIQx and ITCs indeed display lowered mutagenic activity compared to the parent amine. For this purpose, we synthesised a series of unsymmetrical thioureas from MeIQx and the three previously mentioned ITCs whose presence may be expected not only in white cabbage, but also other frequently consumed brassica vegetables. Details of the synthetic procedure and full characterisation of the compounds obtained are included in Supplementary. The results of Ames test MPF presented in Fig. 5 demonstrate that mutagenic potency of synthetic thioureas was indeed lower than that of MeIQx: 3-fold in YG1024 (Fig. 5a) and 6-fold decrease in TA98 strain (Fig. 5b). The higher mutagenicity of conjugates in YG1024 strain, which is especially sensitive towards HAAs, raised doubts regarding the stability of the synthesised compounds under Ames test conditions. It has been demonstrated, that in certain circumstances N,N′-disubstituted thioureas may undergo decomposition to the corresponding amines and isothiocyanates (Drobnica, Kristian, & Augustin, 1977). Consequently, the stability of AITC-MeIQx, PEITC-MeIQx

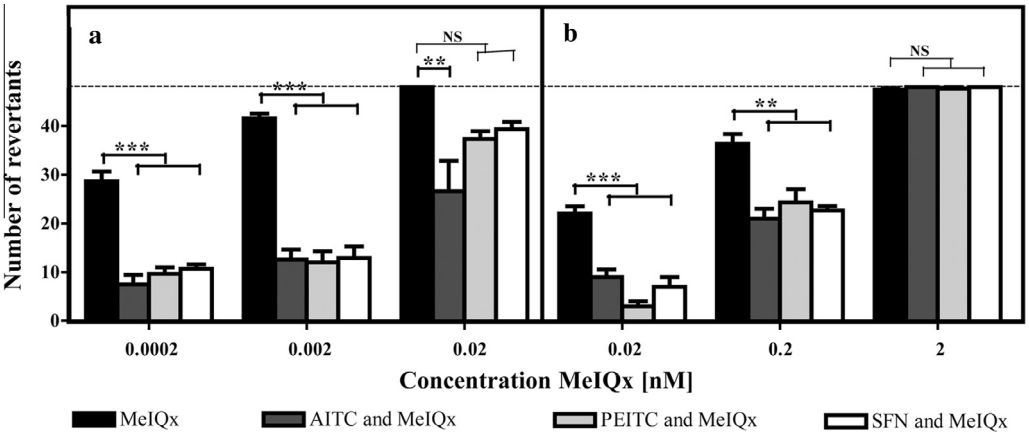

Fig. 4. Mutagenic activity of MeIQx alone or combined with ITCs (determined by a microplate version of Ames test in two *S. typhimurium* strains: (a) YG1024 overexpressing N-hydroxylamine O-acetyltransferase and (b) TA98 standard strain used in Ames test. The concentration of AITC, PEITC and SFN was equal to the highest concentration of MeIQx in the series, *i.e.*, 0.02 nM and 2 nM in experiments involving YG1024 and TA98 strains, respectively. The values are means ± s.e.m. of 9 independent determinations. The statistical analysis was performed by ANOVA with Dunnet's test: **, *** significantly, NS, not significantly, different from MeIQx alone, $^{**}P < 0.01$, $^{***}P < 0.001$.

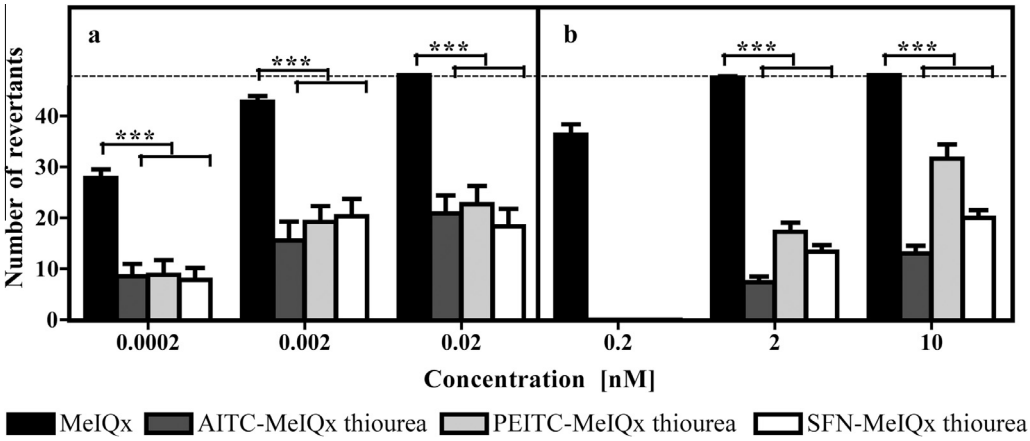

Fig. 5. Mutagenic activity of ITC-MeIQx thioureas determined by a microplate version of Ames test in two *S. typhimurium* strains: (a) YG1024 overexpressing N-hydroxylamine O-acetyltransferase and (b) TA98 standard strain used in Ames test. The values are means ± s.e.m. of 9 independent determinations. The statistical analysis was performed by ANOVA with Dunnet's test: **, *** significantly, NS, not significantly, different from values obtained for MeIQx applied at corresponding concentrations, $^{**}P < 0.01$, $^{***}P < 0.001$.

and SFN-MeIQx was investigated in a simulated Ames test, that is in a solution in which these thioureas were dissolved during mutagenicity determination. The contents of thioureas and MeIQx were monitored in time in the absence (data not shown) or presence of the S9 fraction as presented in Fig. 6. The rate of metabolic decomposition of thioureas was highly increased in the presence of metabolic enzymes present in the S9 fraction; AITC-MeIQx seemed to be least stable and disappeared from the reaction mixture within 30 min and the conjugate of SFN turned out to be most resistant to decomposition. The amount of released MeIQx was equivalent to the concentration of about 0.2 nM, whereas the mutagenicity of MeIQx at this concentration was about 40 revertants per 48

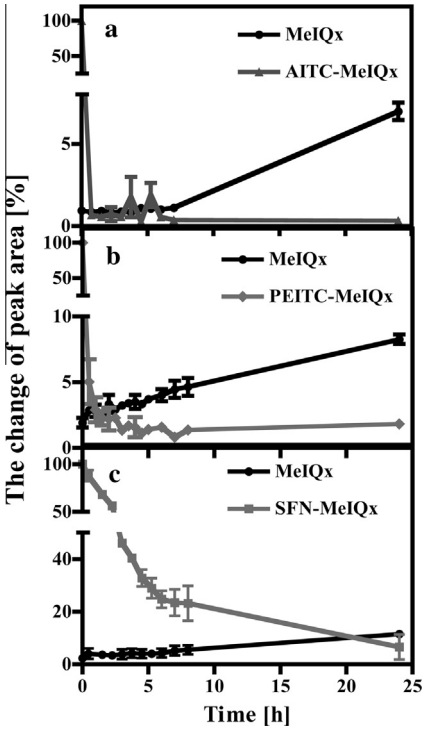

Fig. 6. The decomposition of ITC-MeIQx thioureas and appearance of MeIQx after incubation in Ames test media containing S9 fraction. At times indicated, the samples were collected and the contents of ITC-MeIQx and MeIQx determined by LC–ESI-MS. The values are means ± s.e.m. of 3 independent measurements.

possible, which could explain the observed mutagenicity of thioureas. Still, despite decomposition, the mutagenicity of thioureas was significantly lower than that determined for the parent amine (Fig. 5a and b).

4. Conclusions

The demonstrated antimutagenic activity of ITCs towards HAAs, as a result of chemical detoxification, in addition to a well recognised stimulation of biological detoxification pathways, conforms very well with the results of human intervention studies, in which volunteers were on a diet containing meat processed at a high temperature with highly elevated content of HAAs complemented or not (control group) by mutagen inhibitors, brassica vegetables among them (Shaughnessy et al., 2011). Compared to the control group, in the case of the inhibitor diet, the following protective mechanisms were observed: (i) the increased mutagenicity of hydrolyzed urine suggesting the enhanced conjugation of absorbed mutagens, (ii) decreased mutagenicity of un-hydrolyzed and hydrolyzed feces and (iii) decreased DNA damage in target colorectal cells. These results could be explained by the preventive properties of ITCs, herein proposed. While the first of these observations could be explained by the ability of ITCs to induce in human organism biological mechanisms involved in detoxification of xenobiotics, the latter two would be more convincingly ascribed to chemical detoxification relying on the conversion of HAAs to respective thioureas. Such a conversion would bring about both the decrease in mutagenic activity of excreted HAAs, as well as reduced genotoxicity of these compounds towards cells lining the alimentary tract.

The results of our study may have very important practical implications. The demonstrated improved health quality of burgers prepared from meat mixed with cabbage represents a very simple, inexpensive and easy to implement technique of preparation of thermally processed food products. Such an approach could be adopted in households and food industry (including fast food producers), as well as be exploited for so called anticarcinogenic diets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.01.082.

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