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Naturally occurring diallyl disulfide inhibits the formation of carcinogenic heterocyclic aromatic amines in boiled pork juice

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Three heterocyclic aromatic amines, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoxaline and 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline, have been found in boiled pork juice. We have investigated the effect of naturally occurring organosulfur compounds, which are present in garlic and onion, on mutagen formation in boiled pork juice. Six organosulfur compounds—diallyl disulfide (DAD), dipropyl disulfide (DPD), diallyl sulfide (DAS), allyl methyl sulfide (AMS), allyl mercaptan (AM) and cysteine—were added separately to the pork juice before reflux boiling and then the mutagenicity of each sample was examined with the *Salmonella typhimurium* strain TA98 in the presence of S9 mix. All six compounds were found to inhibit the mutagenicity of boiled pork juice. The greatest inhibitory effect was observed with DAD and DPD, and this was 111-fold higher than that of the lowest, cysteine. To elucidate the inhibitory effect of DAD on mutagen formation in boiled pork juice, the major mutagenic fractions were monitored after HPLC separation by their mutagenicity with *S.typhimurium* TA98. By comparing the retention times of authentic IQ compounds from boiled pork juice with those following the addition of DAD, we showed that the mutagenicity of three major fractions was significantly inhibited compared with those same fractions in boiled pork juice alone. In addition, the Maillard reaction products (MRPs) in the boiled pork juice with and without the addition of DAD were quantified and identified by capillary gas chromatography and gas chromatography–mass spectrometry. The results show that the reduction in the total amount of MRPs (pyridines, pyrazines, thiophenes and thiazoles) in boiled pork juice after boiling for 12 h is correlated with their mutagenicity. Among the MRPs, tetrahydrothiophene-3-one exhibited the strongest correlation. These data suggest that the inhibition of IQ mutagen formation by DAD is mediated through the reduction of MRPs production.

Introduction

Boiling or stewing meat is one of the most common cooking methods in Chinese cuisine, e.g. the dish *Ru-thou* is cooked using ground pork in soy sauce for a relatively long time. Our previous studies have shown that three heterocyclic aromatic amines, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4-dimethyl-imidazo[4,5-*f*]quinoline (MeIQ), are present in boiled pork juice (Lee *et al.*, 1994a). The total amount of IQ-type carcinogens in boiled pork juice is ~4-fold higher than that in broiled beef (Felton *et al.*, 1984, 1986; Lee *et al.*, 1994a). The consumption of carcinogenic heterocyclic aromatic amines from such Chinese foods has not been estimated, but it may be an important factor in the increased incidence of cancers, especially diet-related ones, among Taiwanese people.

Food preparation methods have a significant influence on the formation of heterocyclic aromatic amines, and much research has been devoted to the carcinogens in fried and broiled food (Wong *et al.*, 1982; Jones and Weisburger, 1988; Skog, 1993). Thus, the modification of cooking methods may be helpful in reducing exposure to food-borne carcinogens. In household cooking procedures, meat is frequently cooked with various vegetables, such as garlic and onion, which contain naturally occurring organosulfur compounds in relatively high quantities, such as diallyl sulfide (DAS), diallyl disulfide (DAD), dipropyl disulfide (DPD), allyl methyl sulfide (AMS) and allyl mercaptan (AM) (Block, 1985; Yu *et al.*, 1989). Some of these organosulfur components have been shown to inhibit chemical mutagenesis and carcinogenesis (Sparnins *et al.*, 1988; Knasmuller *et al.*, 1989; Takahashi *et al.*, 1992). However, there is little information regarding their effect on the formation of heterocyclic aromatic amines during the cooking of different meats or in heated model systems. In the present study, the effects of six organosulfur compounds on mutagen formation in boiled pork juice was examined using the Ames test. In addition, the effect of DAD on IQ mutagen formation was determined by HPLC separation and monitoring the mutagenicity of various fractions using the *Salmonella typhimurium* strain TA98. Moreover, the effect of DAD on the formation of MRPs, such as pyridines, pyrazines, thiophenes and thiazoles, which are probably involved in the formation of IQ-type carcinogens in boiled pork juice was also examined. The amount of MRPs produced during the heating process was quantified and identified by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) respectively.

Materials and methods

Chemicals and materials

Lean ground pork was purchased from a local grocery store in Taichung City. Glucose-6-phosphate, nicotinamide dinucleotide phosphate, cysteine and copper phthalocyanine cellulose (blue cotton) were purchased from Sigma Chemical Co. (St Louis, MO). IQ was obtained from Wako Chemical Co. (Tokyo, Japan). MeIQ, MeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline (7,8-DiMeIQx) were kindly provided by Dr S.Grivas (Swedish University of Agriculture Science, Uppsala, Sweden). AM, AMS, tetrahydrothiophene-3-one (THT), pyrazine, 2-methylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-methylpyridine, 2-ethyl-3-methylpyrazine, 2-ethyl-3-methoxypyrazine and 2,4,5-trimethylpyrazine were purchased from Aldrich Chemical Co. (Milwaukee, WI). DAD, DAS and DPD were obtained from Fluka Chemical Co. (Buchs, Switzerland). All other reagents and organic solvents used were of analytic grade.

Effects of organosulfur compounds on the mutagenicity of extracts from a boiling pork system

Boiled pork juice was prepared as previously described (Lin *et al.*, 1982). The condensate, containing 100 g equivalents of fresh ground pork in 25 ml

of pork juice ,was boiled under reflux for 12 h at 102°C. Each organosulfur compound was added separately to the condensate to study its effects on the mutagenicity of the boiled pork juice. Extraction of the mutagenic compounds was performed as previously described (Lin *et al.*, 1982; Lee *et al.*, 1994a). The boiled pork juice with or without the addition of organosulfur compounds was evaluated for mutagenicity using *S.typhimurium* TA98 in the presence of S9 mix.

Effect of DAD on IQ-type carcinogen formation in boiled pork juice

After refluxing, the boiled pork juices with and without DAD were first dissolved in redistilled water and then purified with blue cotton. Details of the procedures were as previously described (Hayatsu *et al.*, 1983). The extract of each sample was dissolved with methanol and subjected to HPLC for further purification (Model D-6500 controlled system, L-6200 gradient pump and L-4500 photodiode array detector, Hitachi Co., Katsata, Japan). In conducting HPLC purification, three steps were used as previously described (Lee *et al.*, 1994a) with a slight modification. Briefly, the sample was first applied to a semi-preparative RP-18 column (7 μm particle, 10×250 mm, Merck) and was eluted at a flow rate of 2.5 ml/min with a gradient of acetonitrile in 10 mM phosphate/sodium hydroxide (pH 7.2). The following concentrations of acetonitrile were used: at 0–5 min, a linear gradient of 10–25%; at 5–10 min, a linear gradient of 25–35%; at 10–20 min, a linear gradient of 35–55%; and at 20–25 min, a linear gradient of 55–60%.

Fractions were collected at 1 min intervals for mutagenicity testing. The mutagenic fractions were then pooled and the material was injected into an RP-18 column (5 μm particle, 4.6×250 mm, E.Merck, Darmstadt, Germany). The mobile phase was acetonitrile/water/diethylamine in the ratio of 12:88:0.1 at a flow rate of 1 ml/min. The fractions were also collected at 1 min intervals to examine their mutagenicity. The mutagenic fraction was finally injected into a Nucleosil CN column (5 μM, 4.6×250 mm, INPAC). The mobile phase was acetonitrile/water/diethylamine in the ratio of 14:86:0.1 at a flow rate of 1 ml/min. The 1 min fractions eluted from HPLC were also tested for their mutagenicity. The four peaks with retention times of 13, 16, 18 and 23 min, corresponding to standard mutagenic compounds MeIQx, DiMeIQx, IQ and MeIQ, were collected separately from a CN column using a photodiode detector and MeIQx, IQ and MeIQ were identified by mass spectrometry according to previous procedures (Lee *et al.*, 1994a). Except for the fraction of DiMeIQx, the UV spectra and mass spectra of the other three peaks correspond to the authentic compounds MeIQx, IQ and MeIQ, respectively (data not shown). The amount of the four IQ-mutagens was determined by their mutagenicity from the corresponding HPLC fractions.

Mutagenicity assays

The samples were tested for their mutagenicity using the plate-incorporation assay with *S.typhimurium* TA98 as described by Maron and Ames (1983). The liver homogenate supernatant (S9) was prepared from the livers of male Sprague–Dawley rats treated with Aroclor 1254 also described by Maron and Ames (1983). A 0.5 ml quantity of S9 mix containing 4% S9 fraction per plate was used for the assay. Each assay was repeated in triplicate and all experiments were performed at least twice. The data were expressed as means ± SD.

GC analysis and GC–MS identification of MRPs in boiled pork juice

The MRPs in the reaction mixtures of boiled pork juice were extracted according to the procedures described by Shibamoto (1982). The GC and GC–MS analysis conditions were conducted using 2,5-dimethyl thiophene as an internal standard to identify different MRPs (Jenq *et al.*, 1994). The capillary GC equipped with a flame ionization detector (Varian Star 3400) was carried out with a 50 m glass capillary (0.32 mm i.d.) coated with CP-WAX 52CB. Conditions were as follows: temperature program 70–200°C at 2°C/min, gas flow 0.8 ml/min of N_2. Mass spectrometric readings were recorded with a JEOL JMS-SX-SX 102A double-focusing instrument using an ionizing energy of 70 eV and ion source temperature of 90°C.

Determination of reducing power

The reducing power of each sample was determined by measuring the absorbance at 700 nm using ferricyanide as a coloring reagent according to the procedures described by Oyaizu (1986).

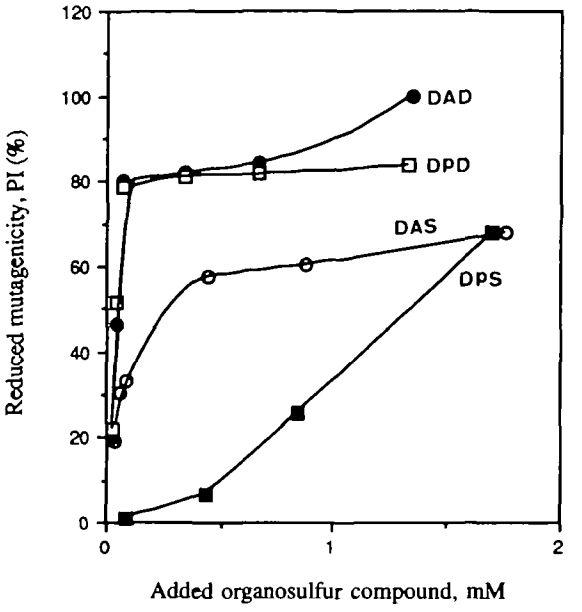
The statistical analysis

The product–moment correlation coefficient, Student–Newman–Keuls test and Student's *t*-test were used for statistical analysis.

Results

Seven organosulfur compounds were added separately to the boiled pork juice before refluxing to determine their effect on the mutagenicity of boiled pork juice extracts (BPE). All compounds showed various dose-dependent responses in reducing the mutagenicity of extracts of boiled pork juice (Figure 1). We calculated the amounts of each organosulfur compound required to inhibit the mutagenicity of BPE by 50% (ID_{50}) from the dose–response curves (Table I) in order to compare their individual inhibitory capabilities. DAD and DPD had the lowest ID_{50} values and showed the highest inhibition. DAS, AMS and DPS had ID_{50} values between 0.1 and 1.0 mM, and showed moderate inhibition. The other two organosulfur compounds, AM and cysteine, showed the lowest inhibition, with ID_{50} values >1.0 mM.

To determine whether the inhibitory effect of organosulfur compounds on the mutagenicity of BPE was mediated through



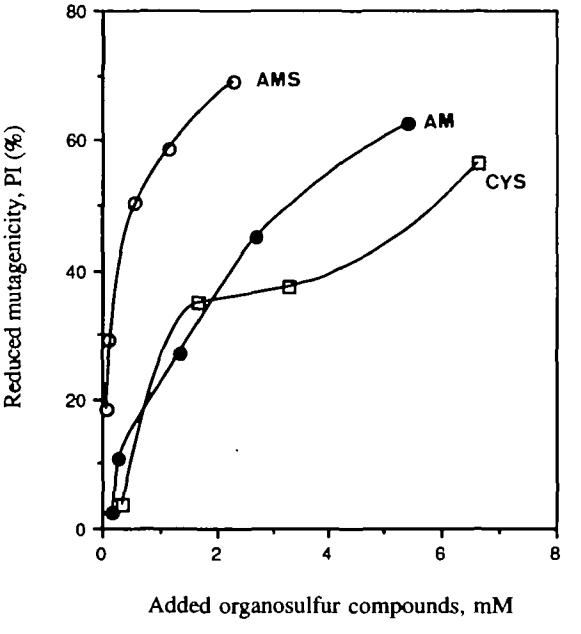


Fig. 1. Inhibitory effect of organosulfur compounds on the mutagenicity of boiled pork juice when added separately before refluxing. Values are percentage inhibition and represent the reduced mutagenicity by organosulfur compounds after refluxing for 12 h. Cys, cysteine.

Table I. Comparison of the inhibitory effect of organosulfur compounds on the mutagenicity of boiled pork juice when they were added separately

Added organosulfur compound	ID_{50}[a] (mM)
DAD	0.05
DPD	0.05
DAS	0.27
AMS	0.49
DPS	1.35
AM	3.47
Cysteine	5.55

[a]The amount of added organosulfur compound required to inhibit the mutagenicity of extracts from boiled pork juice by 50%. The values were calculated from the dose–response curves shown in Figure 1.

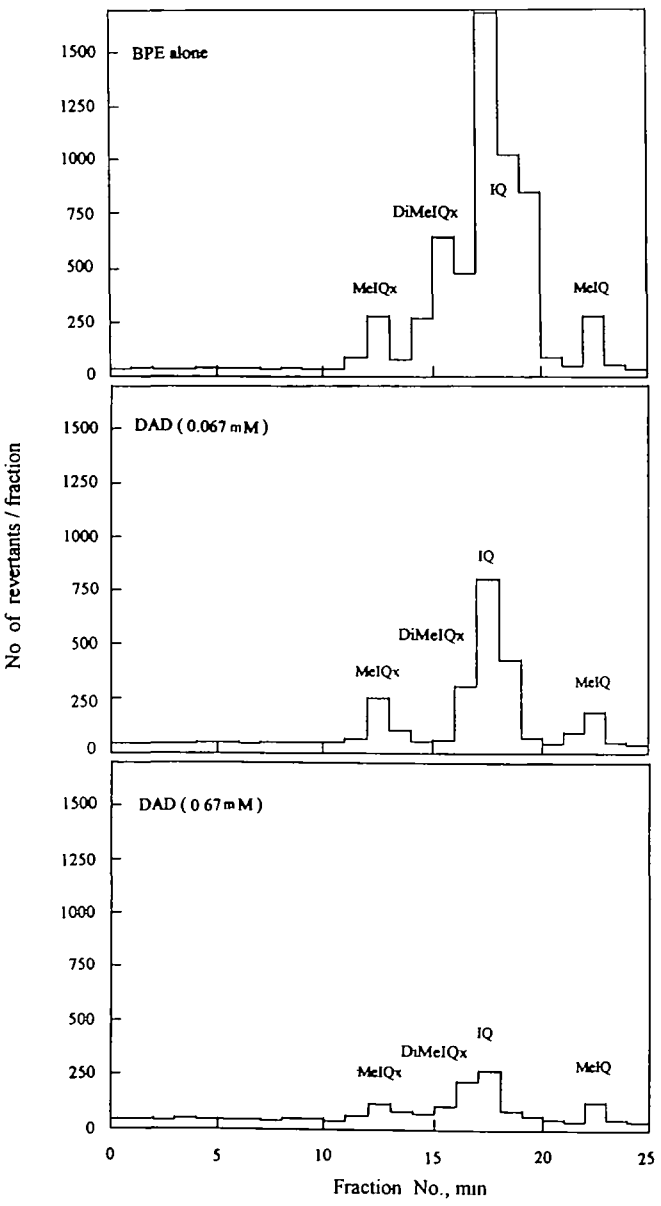


Fig. 2. The mutagenic profiles of 1 min HPLC fractions of boiled pork juice with and without the addition of DAD from a nucleosil CN column.

the reduction of IQ mutagen formation, the mutagenic profiles of HPLC eluents of boiled pork juice with and without the addition of DAD were monitored with *S.typhimurium* TA98

Table II. Effects of the addition of DAD on the formation of mutagens in boiled pork juice

DAD (mM)	Net revertants /fraction[a] (PI, %)[b]			
	MeIQx	DiMeIQx	IQ	MeIQ
0	401	717	4126	318
0.067	283 (29.4)	187 (73.9)	1558 (62.2)	286 (10.1)
0.67	254 (36.7)	135 (81.2)	138 (96.7)	138 (56.6)

The IQ compounds of each major mutagenic fraction were preliminarily identified by the UV spectra compared with its authentic compounds.
[a]Net revertants/fraction: the total net revertants of 1 min fractions of HPLC elutants in each major mutagenic fraction. Data were calculated from the results shown in Figure 2.
[b]The numbers in parentheses are percentage inhibition (PI), which were calculated as follows:

PI (%) = 100 – (the net revertants in the presence of DAD /the net revertants in the absence of DAD)×100.

Table III. The difference in the total amounts of the four major types of MRP in BPE with and without the addition of DAD during a 12 h boiling interval

Boiling time (h)	Changed amounts of MRPs (nmol)[a]				
	Pyridines	Pyrazines	Thiophenes	Thiazoles	Total
1	52.13	14.82	32.75	2.45	102.15
2	42.20	14.26	13.19	4.56	74.21
3	27.30	4.92	10.47	12.92	55.61
4	14.40	4.26	68.88	17.32	104.86
6	12.30	18.35	0.81	13.00	44.46
8	31.31	24.78	2.84	11.41	70.34
10	0.22	17.91	62.28	10.40	90.81
12	1.96	5.35	36.31	5.78	49.40

The total amounts of each major type of MRP in BPE determined by GC–MS contained the following MRPs. Pyridines: 2-methylpyridine; pyrazines: pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine; 2,3-dimethylpyraine, 2-ethyl-3-methylpyraine, 2-ethyl-3-methoxylpyraine, 2-ethyl-5-methylpyrazine, trimethylpyrazine and 1-methyl-3,4-dihydropyrrol(1,2-A)pyrazine; thiophenes: tetrahydrothiophene-3-one and 5-methyl-2-thiophene; thiazoles: thiazole and 4-methylthiazole. Values are means of data from three duplicated experiments.
[a]Change in amounts of MRPs = the amount of MRPs in boiled pork juice without the addition of DAD – the amount of MRPs in boiled pork juice with the addition of DAD (0.67 mM).

in the presence of S9 mix. The mutagenic profiles of boiled pork juice with the addition of DAD were similar to those of boiled pork juice alone (Figure 2). However, the mutagenicity of four of the major fractions corresponding to the retention time of authentic IQ compounds (MeIQx, DiMeIQx, IQ and MeIQ) in boiled pork juice was decreased by the addition of DAD. Table II shows that the mutagenicity (e.g. the number of revertants) of those HPLC fractions corresponding to DiMeIQx and IQ were reduced more than those corresponding to MeIQx and MeIQ. A dose-dependent inhibitory effect of DAD on the four major mutagenic HPLC fractions was observed. Thus, the addition of DAD reduces the mutagenicity of boiled pork juice, by inhibiting IQ mutagen formation.

To elucidate the mechanism of DAD inhibition on IQ mutagen formation in boiled pork juice, the amounts of MRPs (including pyridines, pyrazines, thiophenes and thiazoles), which we have previously reported to be involved in the formation of IQ mutagens (Jagerstad *et al.*, 1991; Lee *et al.*, 1994b), were analyzed by GC and GC–MS after boiling for

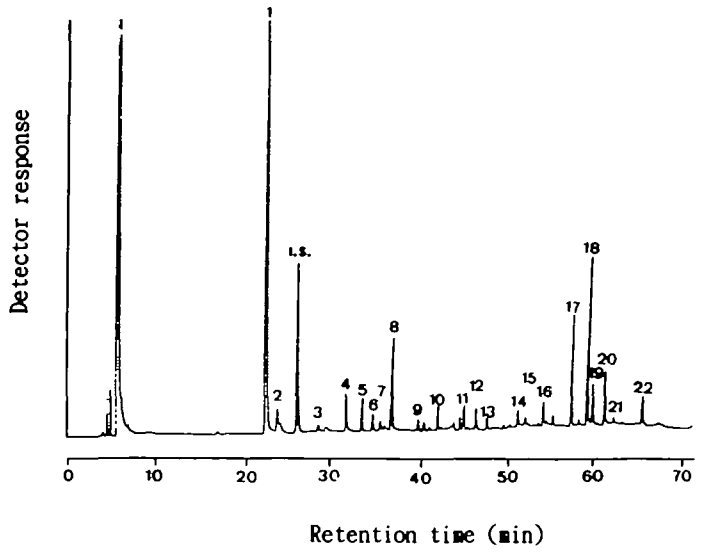


Fig. 3. The GC profile of MRPs extracted from pork juice boiled for 12 h. All MRPs were identified by GC–MS and quantified using 2,5-dimethylthiophene as an internal standard. Peak 1, thiazole; 2, 5-methyl-2-thiophene; 3, 2-methylpyridine; 4, pyrazine; 5, 2-methylpyrazine; 6, 4-methylthiazole; 7, 2,3-dimethylpyrazine; 8, 2,5-dimethylpyrazine; 9, 2,6-dimethylpyrazine; 10, trimethylpyrazine; 11, 2-ethyl-3-methylpyrazine; 12, 2-ethyl-5-methylpyrazine; 13, 2-ethyl-3-methoxylpyrazine; 14, 1-methyl-3,4-dihydropyrrol(1,2-A)pyrazine; 15, sulfinylbiomethane; 16, 1,4-diethylpyrazole; 17, 2-furanethamol; 18, prapandiamine; 19, tetrahydrothiophene-3-one, 20, 2-hydroxy-3-methyl-2-cyclopentene-1-one; 21, unknown; 22, unknown; I.S., internal standard (2,5-dimethyl thiophene).

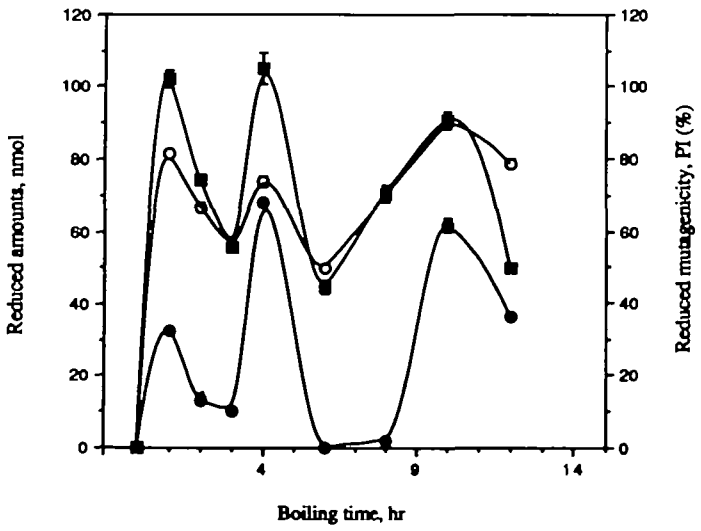


Fig. 4. The correlation between the reduced amount of THT (●) and total MRPs (■) (including 2-methylpyridine, pyrazines, thiophenes and thiozoles) in boiled pork juice and their mutagenicity with different boiling times (○). Five percent of the total extracts per plate were used for the mutagenicity test. The assay was examined with *S.typhimurium* TA98 in the presence of S9 mix. Values of percentage inhibition represent the reduced mutagenicity of boiled pork juice by DAD after refluxing for 12 h. The product–moment correlation coefficient, Student–Newman–Keuls test and Student's *t*-test were used for statistical analysis. The reduced amounts of THT and total MRPs correlated with a fall in mutagenicity ($r = 0.64$, $P < 0.05$ for THT; $r = 0.87$, $P < 0.01$ for the total MRPs).

various times. The four major MRPs were determined in boiled pork juice with and without the addition of DAD every 1–2 h over a total boiling period of 12 h. Five other MRPs and one

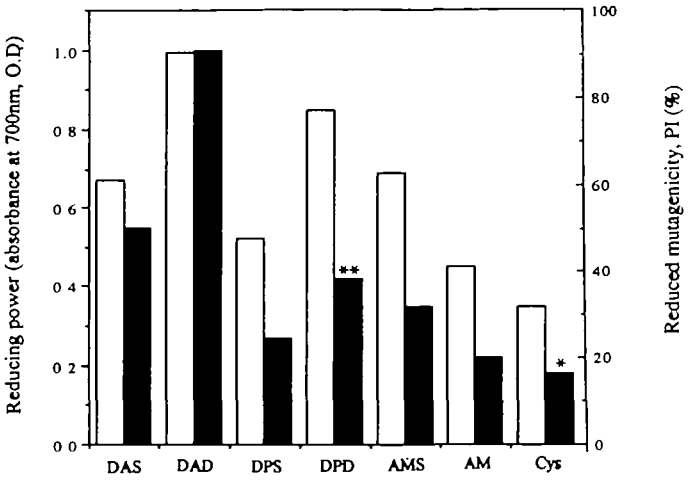


Fig. 5. The correlation between the reducing power of six organosulfur compounds and their inhibitory effect on the mutagenicity of boiled pork juice when added separately. Values of percentage inhibition also represent the mutagenicity of boiled pork juice by DAD after refluxing for 12 h. Reducing power, □; reduced mutagenicity, ■. The product–moment correlation coefficient and Student's *t*-test were also used for statistical analysis ($r = 0.80$, $P < 0.01$). *$P < 0.05$; **$P < 0.01$.

unknown compound were also examined, but these represented <5% of total MRPs (data not shown). Table III shows that the amounts of the four major MRPs after various boiling intervals were reduced by the addition of DAD compared with the amounts in boiled pork juice alone. Among them, thiophenes showed the greatest reduction, followed by the pyridines, pyrazines and thiazoles.

The correlation between the reduced amounts of each type of MRPs and the mutagenicity of boiled pork juice after various boiling intervals was analyzed statistically by linear regression. The results indicate that overall, the reduced amounts of THT and the four major MRPs correlated with a fall in mutagenicity (Figure 3) ($r = 0.64$, $P < 0.05$ for THT; $r = 0.87$, $P < 0.01$ for the four major MRPs). However, when each MRP was examined alone, there was no correlation with BPE-mutagenicity. These results suggest that THT may play an important role in IQ-mutagen formation in boiled pork juice. In addition, the reducing power of the seven organosulfur compounds showed a good correlation with their inhibitory effects on the mutagenicity of boiled pork juice (Figure 4; $r = 0.80$, $P < 0.01$). This indicated that the reduced amounts of MRPs in boiled pork juice after the addition of DAD may be due to it having the highest reducing power among the seven organosulfur compounds.

Discussion

In our previous study we added six MRPs separately to pork juice before reflux boiling and our results suggested that two MRPs, 2,3-dimethylpyrazine and THT, may be involved in the formation of IQ-type mutagens in boiled pork juice (Lee *et al.*, 1995). In the present study, we found that IQ-mutagen formation and MRP production in boiled pork juice were inhibited by the addition of naturally occurring organosulfur compounds such as DAD. Among the MRPs affected, THT showed the greatest reduction. These results further suggest that THT may play an important role in IQ-mutagen formation in the reflux boiling of pork juice extracts.

Although we have not investigated the content of IQ-mutagens in stewed pork, there are reports that some food-grade beef extracts contain low levels of IQ-mutagens (Aeschbacher *et al.*, 1987; Jackson *et al.*, 1994). In normal Chinese household cuisine, 3–5 pieces of garlic (7.5–12.5 g) or 1–2 onions (300–500 g) are cooked with meat. The amounts of DAD in garlic and onion were estimated to be ~1.98–3.30 and 0.47–0.78 mM respectively (Yu *et al.*, 1989). Our data indicate that 50% of BPE-mutagenicity (100 g) can be reduced by the addition of 0.05 mM DAD (Table I). Thus, using DAD to prevent the formation of IQ-mutagens in everyday cooking is feasible.

Friedmen and Molnar-Perl (1990) have previously shown that sulfur-containing amino acids such as cysteine, *N*-acetyl-cysteine and reduced glutathione, greatly inhibit non-enzymatic browning of heated amino acid–glucose mixtures. However, in our study the inhibitory effect of cysteine on mutagen formation via the Maillard reaction in boiled pork juice was ~100-fold lower than that of the naturally occurring organosulfur compounds we tested, with the exception of AM (Table I). The nature of the inhibition of Maillard reaction by sulfur-containing amino acids is still not well understood. However, two of the mechanisms postulated are (i) suppression of free-radical formation through trapping by thiols in sulfur-containing amino acids (Friedman, 1984; Friedman and Molnar-Perl, 1990) and (ii) interaction of the sulfhydryl compounds with intermediates formed during browning, thus trapping and/or preventing them from forming the final MRPs (Friedman and Molnar-Perl, 1990). The Maillard reaction which occurs during food processing is known to be suppressed by the addition of antioxidants (Namiki, 1988). Our data indicate that the reducing power of the organosulfur compounds tested correlated well with their inhibitory effects on the mutagenicity of boiled pork juice (Figure 4). This result suggests that the reduction of BPE-mutagenicity may be attributed to their antioxidative properties. Whether or not the organosulfur compounds exert their effect by scavenging free radicals needs further investigation. However, it is known that in the presence of a thiol, aldehydes competitively interact with one or two SH groups to form a thiohemiketal or thioketal, thus blocking the Maillard reaction (Friedman and Molnar-Perl, 1990). Friedman (1991) proposed a similar mechanism to explain how sulfhydroxy-containing amino acids, such as β-alanine, *N*-α-acetyl-L-lysine and L-cysteine, minimize the Maillard reaction. The present study showed that the production of MRPs (including pyridines, pyrazines, thiophenes and thiazoles) in boiled pork juice with the addition of DAD was decreased by 46–77% during the 12 h boiling interval compared with boiled pork juice alone (Table III). The inhibition of the Maillard reaction in boiled pork juice by DAD may be explained by one or both of the mechanisms described here.

Recently a hypothesis first proposed by Jagerstad *et al.* (1983, 1991) for the formation of IQ compounds via the Maillard reaction has been defined. Milic *et al.* (1993) demonstrated that MeIQx and MeIQ were formed after heating aqueous 2,5-dimethylpyridine (DMP) or 2-methylpyridine with creatine and acetaldehyde. We have recently reported evidence for the formation of IQ in a model system in which 2-methylpyridine, acetylformaldehyde and creatinine were heated in diethylene glycol containing 5% water for 1 h at 140°C (Lee *et al.*, 1994b). However, these studies do not reflect the complexity of model systems such as boiled pork juice and boiled beef juice. Taylor *et al.* (1986) reported that the addition of five pyrazines did not change the mutagenicity of boiled beef extracts. In the present study, our data indicated that the reduced amounts of 2-methylpyridine and 10 pyrazines in boiled pork juice following the addition of DAD to the boiled pork juice reflux mixture did not correlate with its mutagenicity. However, we did observe a reduced amount of THT in boiled pork juice with added DAD, and this correlated with BPE-mutagenicity. These results imply that THT is more important than pyridines and pyrazines in the formation of IQ-mutagens in boiled pork juice. A recent report has shown that aldehyde acts as a key reactant in IQ-mutagen formation (Lee *et al.*, 1994b). Interestingly, we have observed that THT seems to cause the production of aldehydes after heating aqueous 2-Mp or 2-methylpyridine with creatinine and THT (S.N.Jenq, S.-J.Tsai and H.Lee, unpublished data). Therefore, the possible role of THT in the formation of IQ compounds mediated through the formation of aldehydes must be further investigated. Furthermore, studies on the mechanisms involved in the inhibitory effects of organosulfur compounds on IQ-mutagen formation should provide an insight into efficient ways to reduce their formation.

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