CHAPTER 4

DISCUSSION

Methanol extract of shallot (Allium ascalonicum Linn.) showed mutagenicity toward TA98 stronger than TA 100. This indicated that the extract has strong framshift mutagenesis than base-substitution mutagenesis. The mutagenicity was in the same pattern as in the studies of Purintrapiban (Purintrapiban,J.,1989) and Vinitketkumnuen (Vinitketkumnuen, U.,1991) indicating the presence of naturally occurring mutagenic substances in shallot. The varying potentcy of mutagenic activity might be due to different amount of the mutagenic substances in shallot samples. The mutagenicity of shallot indicated the presence of direct acting mutagen(s) without metabolic activation (S9 mix). However, after the addition of S9 mix, the mutagenicity was increased, due to the microsomal enzymatic activation.

4.1 Factors modifying shallot mutagenesis.

The biological effect of mutagen may depend upon the concentration of its active form and the duration of that form inside the body. This is governed, in turn, by the rate of its biotransformation and excretion and the magnitude and nature of its binding to tissue macromolecules.

Mutagens, once inside the body, undergo a various biotransformations. Those reactions that introduce a new functional group into the molecule, either by oxidation, reduction or hydrolysis, are designated as phase I

reactions while the conjugation reactions by which phase I metabolites are combined with endogenous substrates in the body one referred to as phase II reaction.

Salmonella mutation assay has been widely used for toxicological evaluation in evaluating the risk of environmental chemicals for man (Ames, B.N., et al, 1973, Matsushima, T. et al. 1980). This technique has been modified to develop in vitro conditions that would mimic the type of metabolic conversion that occurs in vivo. A wide range of factors have been found to affect the sensitivity of the in vitro technique (Sugimura, T., 1988). The Salmonella mutation test usually evaluated only the activated compounds by adding mammalian S9 fraction (i.e. rat liver S9 fraction) which contains the cytochrome P-450 system to activate the compounds. Activation may be only a part of the chemical's in vivo metabolism, the activated compounds may undergo conjugation (phase II reaction) by endogenous compounds (i.e. GSH). Modifying the assay by incorporating the conjugation reaction could provide valuable information.

Mutagens in shallot was highly mutagenic to Salmonella typhimurium TA 98. The mutagens are metabolized by enzymes in S9 fraction to form more active intermediates with higher mutagenicity (ultimate mutagens). The intermediates can form adducts with DNA and other cellular macromolecules or can undergo conjugation with GSH or other conjugating agents, which is the form to be excreted.

This investigation demonstrated that shallot mutagenesis was affected by the presence of GSH. At 14.8 mM GSH decreased the mutagenicity of shallot extract in TA 98 and, TA 100, with and without liver S9 fraction as shown in Fig. 7. At concentrations of 4 mg methanol extract of shallot, GSH significantly decrease the shallot mutagenesis (85 % reduction). At concentrations above 4 mg i.e., 20 mg of the extracted, the inhibition was slightly decreased (23 % reduction) as shown in Table 2. It was suggested that glutathione might directly react with mutagenic substances in shallot. Chemical reaction between sulfhydryl group of GSH and functional group of mutagenic substances of shallot might inactivate the mutagenic substances. At low concentrations of the extract, reaction with sulhydryl group resulted in less available of functional groups of mutagenic substances to extert mutagenesis ; at higher concentration there might be a saturation of the GSH, excess mutagenic substances were still available for mutagenesis.

The effect of 14.8 mM GSH on the mutagenicity of shallot extract in the presence of liver S9 fraction was significantly decreased. The mutagenicity was inhibited to a greater extent by the addition of liver S9 fraction. A reaction of the mutagenicity of shallot extract by conjugation with GSH in the presence of liver metabolic active fraction might be expected. The conjugation to glutathione has been implied in the detoxification of mutagenic substances in shallot.

The effects of incubating shallot extracted prior to mixing with bacteria, with GSH in the presence of S9 mix, was demonstrated in Fig. 20. It confirmed that the possible mechanism of GSH deactivation could include not only direct reaction with the mutagens, but also undergo conjugating reaction with GSH. Glutathione S-transferase, enzymes which catalysed this reaction has been found in rat liver microsomal enzyme (Waziers, I.D., and Decloitre, F., 1984).

The conjugation to UDP-glucuronide (UDPGA) is known to represent another major detoxication pathways in mammals. (Kasper, C.B., and Henton. D., 1980). This investigation also demonstrated reduction in mutagenicity of shallot extract after the addition of UDPGA in the presence of liver S9 fraction. The reduction of shallot mutagenicity by the addition of UDPGA was statistically significant. The unchanged mutagenicity of shallot extract after the addition of UDPGA in the absence of liver S9 fraction indicatd that UDPGA might not be able to react directly with mutagenic substances in shallot extract.

4.2 Modulation of the mutagenicity of shallot extract.

Great interest has developed during the past few years in the ability of natural factors to modify the metabolic activation of environmental mutagens. This knowledge is important for evaluating the hazards that these compounds are able to inflict on humans.

This investigation sought to find the factors able to modify the bacterial mutagenicity of shallot extract.

Retinoids or vitamin A play an important role in chemical carcinogenesis(Elias, P.M., and Williams, M.L., 1981 and Goodman, D.S., 1979). Retinoids have the ability to inhibit the mutagenicity of precarcinogens only (Balbinder, E.,et al,1983). It has been suggested that retinoids might extert their anti-tumor or anti-mutagenic activity on the metabolic activation of a precarcinogen, probably by inhibiting the enzyme activities required for metabolic activation (Qin, S., and Huang, C.C., 1985).

The results on the effect of retinoic acid on the induction of His^+ revertant by methanol extract of shallot is shown in Fig. 12. It was demonstrated that retinoic acid could not affect the mutagenicity of shallot extract to Salmonella typhimurium strains TA 98 and TA 100 with and without liver S9 fraction.

Ascorbic acid has been reported to decrease mutagen formation in canned salmon (Meester, C.D., 1989). An effect of ascorbic acid on shallot mutagenicity in Salmonella mutation assay was studied in this thesis. The result indicated that ascorbic acid did not, however, affect the mutagenicity of shallot extract to Salmonella mutation. It might be postulated that ascorbic acid did not have a comutagenic effect.

Activation of mutagenic substances in shallot extract was observed after treatment with nitrite under acidic conditions. The presumed nitrosation products showed mutagenicity to TA 100 in the presence and absence of S9 mix. This suggested possible nitrosation precursor presented in shallot extract.

Nitrate and nitrite in drinking water and food are commonly consumed among Thai people. Moreover, poor oral hygiene, nitrate in saliva can be converted to nitrite by oral microflora (Tannenbaum, S.R., et al, 1976). Nitrosation compounds might be formed from mutagenic substances of shallot and nitrite in our body. Shallot consumption will not only consider its direct mutagenicity, but also its nitrosation precursor presentation.

Another sulfhydryl-containing compound ,dithiothreitol (DTT) decreased mutagenicity of shallot extract as shown in Fig. 9 and Fig. 10. An addition of liver S9 fraction did not enhance the inhibition potency of dithiothreitol.

The possible mechanism of DTT inactivation could include only direct reaction with mutagens in shallot extract.

4.3 Modulation of quercetin mutagenesis.

Quercetin, a plant flavonoid, has been reported to play an important role in the mutagenicity of shallot (Vinitketkumnuen, U., 1991). The modulation of quercetin mutagenicity by the same above-mentioned chemicals was also studied. As shown in Fig. 17 GSH can suppress the

mutagenicity of quercetin. It can be postulated that this similar inhibitory effect of GSH could be explained by direct reaction due to SH group of GSH with functional group of quercetin and by glutathione conjugation.

SH-containing compounds such as GSH, DTT and cysteine could reduce the mutagenicity of shallot as shown in Fig. 18 and Fig.19. Presumably, the primary interaction in these compounds occurs via SH-group.

4.4 Partial purification of shallot extract.

Partial purification of active mutagenic principles in shallot extract was performed by SEP-PAK C_{18} Cartridge. Active mutagenic principles were isolated into two fractions, 50 % methanol eluent and 100 % methanol eluent. Both of them were further isolated into many fractions by Sephadex LH-20 chromatography. The 50 % methanol eluent was fractionated into 4 peaks. The mutagenic activity to S. typhimurium TA 98 was demonstrated in the last major peak (peak IV) ofthe chromatogram (Fig. 23). This peak IV was further chromatographed into one prominent mutagen peaks (peak IV_{a2}) which gave a yield of about 0.22 µg per 1 mg shallot extract. The isolated mutagenic compound also showed a single peak that corresponded, by the elution time, to pure quercetin.

However,the co-chromatography of the unknown compound (peak IV_{a2}) by Sephadex LH-20 with authentic quercetin did not show an enough increase of the peak height (Fig. 28 C) as theoretically expected or calculated from A and B. The small peak shoulder appeared in C might be due to some impurity present in the partially purified fraction.

As for the UV-spectra pattern of various pure flavonoids, i.e. quercetin, quercetrin and flavanone shown in Fig. 29 b-d, the flavonoids have their common characteristic patterns of UV-absorption spectra. The patterns have two maximum wavelengths, one peak at 300-400 nm and another at 220-260 nm. The UV-absorption pattern of the partially purified peak IV_{a2} (Fig.29 a) was similar to that of authentic quercetin (Fig. 29 b), but different from those of quercetin glycoside (Fig. 29 c) and flavanone (Fig. 29 d). The unknown mutagenic compound showed two main peaks (230 nm and 370 nm) and a small shoulder at the first peak which are closely similar to those of quercetin. Therefore spectroscopically, it is suggested that the main mutagenic compound might be likely a quercetin or any other compound in a group of quercetin glycosides.

Since the biological effects of GSH and DTT on Salmonella mutagenicity of the shallot extract are similar to that of pure quercetin either in the presence or absence of S9 mix, it indicates that the shallot mutagenic

compound(s) genotoxically behaved on bacteria like quercetin and therefore, it is presumed that the mutagenic activity of shallot could be due to the presence of quercetin or quercetin-like compound. The presence of metabolic activation could increase the mutagenic activity of quercetin, explained according to MacGregor's suggestion (MacGregor,J.T., 1984), that hydroxyl groups of quercetin are oxidized to unknown ultimate reactive metabolites. This might be the same as in the unknown mutagenic compound from shallot.

In this study, the specific mutagenic activities of quercetin, the peak IV_{a2} compound from shallot and quercetrin are 35,300 ,10,109 and 940 His^+ revertant colonies/mg , respectively. They were all tested by S. typhimurium TA 98 in the presence of S9 mix. Comparatively, the specific mutagenic activity of the peak IV_{a2} compound is stronger than quercetrin (11 folds) but less than quercetin (3.5 folds). The reduction of specific mutagenic activity of the shallot mutagen compound, as compared to that of quercetin, might be due to its glycosidic forms. The shallot mutagen might be quercetin monoglycoside,i.e. quercetin-7-glucoside, quercetin-3´-glucoside or quercetin-4´-glucoside but not quercetrin. These quercetin monoglycosides were reported to be weakly mutagenic to S. typhimurium (Vinitketkumnuen,U.,1991).

Further chromatography by Sephadex LH-20 column chromatography using 100 % methanol eluent also demonstrated the presence of mutagens (the last peak, M-6). However, final purification of this mutagenic peak was not possible. The amount of isolated peak was too small and was therefore not sufficient to allow continue purification in this study.