

## Enhancement of the Mutagenicity of Trp-P-1, Trp-P-2 and Benzo[a]pyrene by Bupleuri Radix Extract

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Boiling-water extract of Korean-Saiko (Bupleuri Radix, from South Korea, *Bupleurum falcatum* L.) enhanced the mutagenic activity of Trp-P-1, Trp-P-2 and benzo[a]pyrene with S9mix. The boiling-water extract was fractionated with ether and then *n*-BuOH. Both the ether and the *n*-BuOH fractions also enhanced mutagenicity of Trp-P-1, respectively. The *n*-BuOH fraction was separated into seven fractions by silica gel chromatography and the chloroform eluate had the strongest enhancing effect on the mutagenic activity of Trp-P-1 with S9mix.

The chloroform eluate fraction was further separated into five spots by thin-layer chromatography. Two of the spots had the strongest enhancing effect on the mutagenic activity of Trp-P-1.

Since saikosaponin is a well known component in Bupleuri Radix, the effects of its existence were tested and saikosaponins a and c were found. The enhancement activity of saikosaponin a was very weak. The effective components are now being studied.

**Keywords** Bupleuri Radix; Saiko extract; saikosaponin; enhancement; mutagenicity; Ames assay; benzo[a]pyrene; Trp-P-1; Trp-P-2; activated Trp-P-1

### Introduction

Recently antimutagens have been found in crude drugs such as Rhubarb Radix,<sup>1)</sup> *Zingiber officinale*,<sup>2)</sup> Isodonis Herba,<sup>3)</sup> Cinnamomi Cortex,<sup>4)</sup> Ranunculus and Anemone plants<sup>5)</sup> and *Glycyrrhiza glabra*.<sup>6)</sup>

However, reports relating to the enhancement effect of crude drugs on mutagenic activity have been very few. Only the effect of quercetin on the mutagenicity of 2-acetylaminofluorene and benzo[a]pyrene has been reported by Ogawa *et al.*<sup>7)</sup>

We have been studying to find the antimutagenic substances in crude drugs. In the experiment, enhancement effects on the mutagenic activity of benzo[a]pyrene was found in Bupleuri Radix. It is very important because the use of Chinese drugs has been increasing. So, in this paper the enhancement effect on the mutagenic activity of Trp-P-1, Trp-P-2 and benzo[a]pyrene were studied. Evaluation of the enhancement activity was carried out by using the Ames system.<sup>8)</sup>

### Results

**Enhancement Effect on Mutagenic Activity** Boiling-water extracts of five kinds of Bupleuri Radix enhanced the mutagenic activity of Trp-P-1 for *Salmonella typhimurium* TA98, as shown in Fig. 1.

A dose-response relationship was recognized above 0.5 mg/plate, and especially Tianjin-Saiko and Japan-Saiko B appeared to have a strong effect.

Boiling-water extract from Korean-Saiko enhanced the mutagenicity of Trp-P-2 for TA98 and of benzo[a]pyrene for TA98 and TA100. These results are shown in Fig. 2.

Dose-response relationships were observed on Trp-P-2 and benzo[a]pyrene for strain TA98. But for strain TA100, enhancement on the mutagenicity of benzo[a]pyrene was recognized at only 25 mg/plate.

The boiling-water extract did not show mutagenicity and the number of surviving colonies was not affected by any concentration of boiling-water extract. Therefore, it is suggested that enhancement factor(s) existed in the boil-

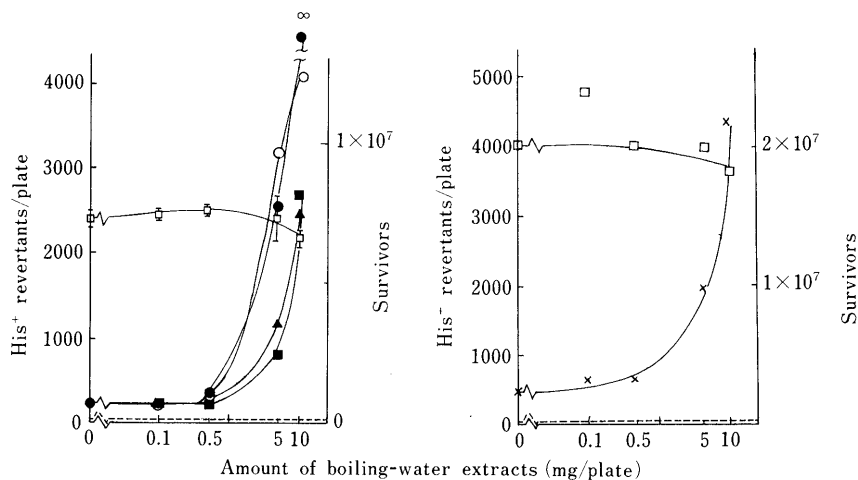


Fig. 1. Effect of Four Kinds of Boiling-Water Extracts on the Mutagenicity of Trp-P-1

0.5  $\mu$ g/plate of Trp-P-1 was added for the strain TA98 with S9mix. ●—●, Tianjin-Saiko (*Bupleurum chinensis* D.C.); ○—○, Sho-Saiko (from Kohokusho in China, *Bupleurum tenue* BUCH.-HAM. *ex* D. DON); ■—■, Korean-Saiko (*Bupleurum falcatum* L.); ▲—▲, Japan-Saiko A (from Kumamoto-prefecture, *Bupleurum falcatum* L.); ×—×, Japan-Saiko B (from a herbal garden at Gifu Pharmaceutical University, *Bupleurum falcatum* L.); □—□, survival colonies that were due to S.E. ----, spontaneous mutation.

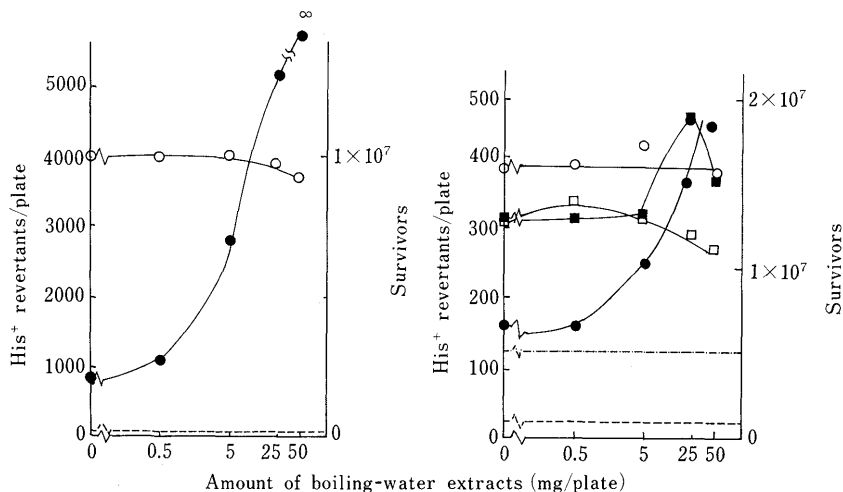


Fig. 2. Effect of Boiling-Water Extract of Korean-Saiko on the Mutagenicity of Trp-P-2 (Left) and of Benzo[a]pyrene (Right)

0.2  $\mu$ g/plate of Trp-P-2 was added for the strain TA98. 5  $\mu$ g/plate of benzo[a]pyrene was added for the strain TA98 and TA100 with S9mix. ●—●, revertant colonies on TA98; ■—■, revertant colonies on TA100; ○—○, survival colonies on TA98; □—□, survival colonies on TA100; ----, spontaneous mutation of TA98; - - - - , spontaneous mutation of TA100.

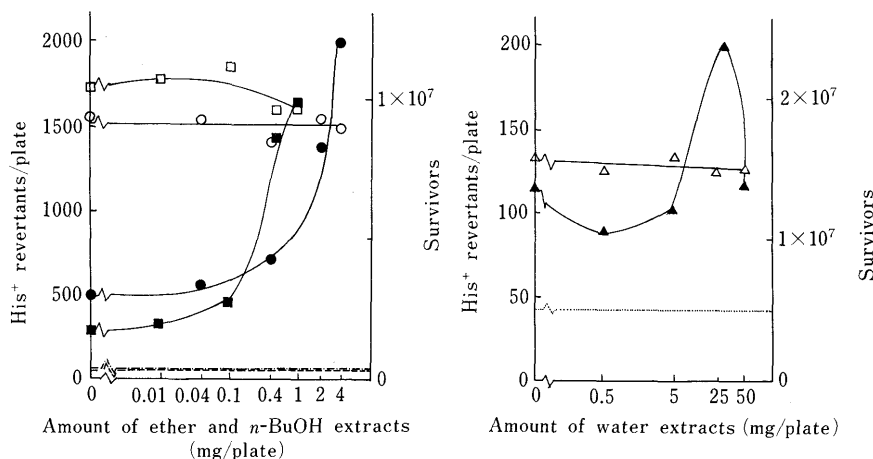


Fig. 3. Effects of Ether, *n*-BuOH and Water Fractions on the Mutagenicity of Trp-P-1

0.2  $\mu$ g/plate of Trp-P-1 was added for the strain TA98 with S9mix. Revertant colonies of each fraction are as follows; ether fraction (■), *n*-BuOH fraction (●), water fraction (▲), and survival colonies of each fraction are expressed as the same open symbols. Spontaneous mutation of each fraction is as follows; ---, ether fraction; - - - - , *n*-BuOH fraction; - - - - , water fraction.

ing-water extract of Bupleuri Radix.

**Separation of Active Substances** In the procedure for separation, the activity was tested to find out the effective fractions. Trp-P-1 was used as the testing chemical, because the results were nearly the same between Trp-P-1 and Trp-P-2, and the activity was clearly shown.

The results of Ames assay for ether, *n*-BuOH and water fractions are shown in Fig. 3.

Both ether and *n*-BuOH layers enhanced mutagenicity of Trp-P-1. Dose-response relationships were observed in the range of 0.1—1 mg/plate for the ether layer, and 0.4—4 mg/plate for the *n*-BuOH layer. Residue of water layer enhanced the mutagenicity of Trp-P-1 twice only at 25 mg/plate. Each amount of fraction, separated from 1.8247 g of *n*-BuOH extract, converted into from 20 mg of *n*-BuOH extract, and each corresponding amount was tested. Revertants by the whole amount of each fraction are shown in Fig. 4.

The number of revertants by frs. 1, 2 and 3 were more than 3 times that by Trp-P-1 when their enhancing abilities

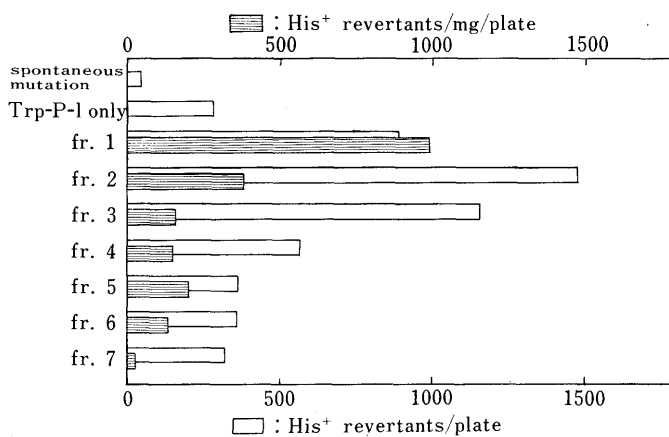


Fig. 4. Effects of Each Fraction of *n*-BuOH Extract Separated by Silica Gel Chromatography on the Mutagenicity of Trp-P-1

0.2  $\mu$ g/plate of Trp-P-1 was added for the strain TA98 with S9mix. ▨, the number of revertants by each fraction were calculated by following the equation: revertant colonies =  $\frac{\text{sample} - (\text{N.C.} + \text{P.C.})}{\text{sample (mg)}}$  N.C., spontaneous mutation; P.C., Trp-P-1 only. □, revertants by each fraction obtained 20 mg of *n*-BuOH extracts.

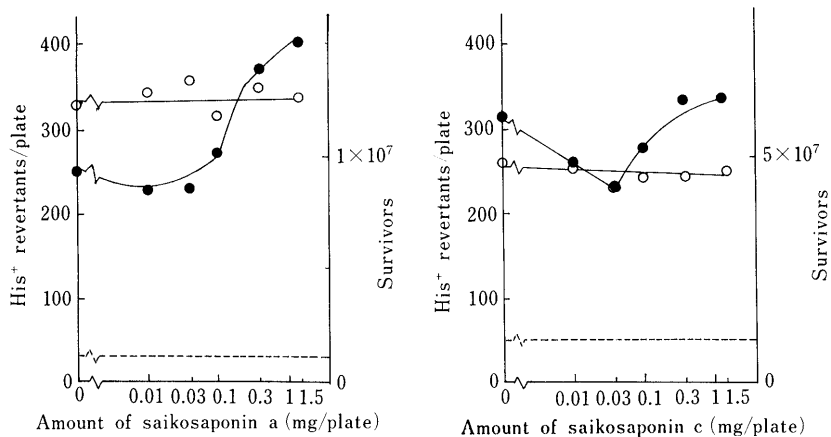


Fig. 5. Effects of Saikosaponins a and c on the Mutagenicity of Trp-P-1

0.2 μg/plate of Trp-P-1 was added on the strain TA98 with S9mix. ●—●, revertant colonies; ○—○, survival colonies; ---, spontaneous mutation.

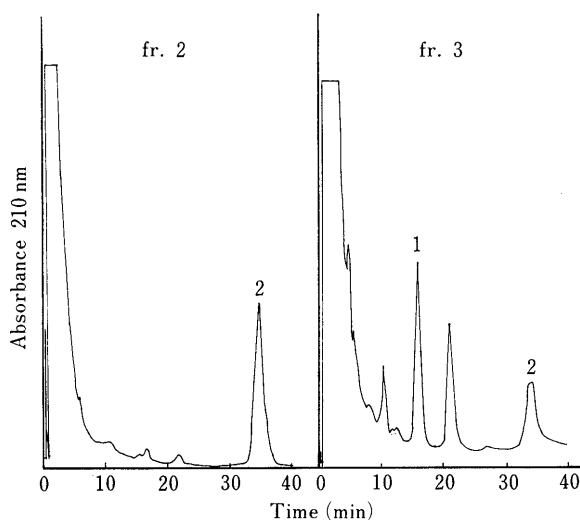


Fig. 6. High Performance Liquid Chromatograms of Fr. 2, 3 of *n*-BuOH Extracts Separated by Silica Gel Chromatography

Peaks: 1, saikosaponin c; 2, saikosaponin a. Column: Wakogel-column, ODS-10K (4 mm i.d. × 30 cm). Eluent: CH<sub>3</sub>CN-H<sub>2</sub>O (35:65). Flow-rate: 2 ml/min. Sample injection: 3 μl.

per 1 mg of each fraction were calculated by following the Eq. 1.

$$\text{revertant colonies/mg} = \frac{\text{sample} - (\text{N.C.} + \text{P.C.})}{\text{sample (mg)}} \quad (1)$$

(N.C.; spontaneous mutation, P.C.; Trp-P-1 only) Revertants/mg/plate of fr. 1 was about 1000 and the strongest ability was found.

On thin-layer plate, the existence of saikosaponins in each fraction was tested and saikosaponins a and c were found in frs. 1, 2 and 3.

The activity of saikosaponins a and c was tested as shown in Fig. 5. Saikosaponin a (1.5 mg/plate) enhanced mutagenicity of Trp-P-1 1.5 times and showed a dose-response relationship, saikosaponin c did not enhance mutagenicity of Trp-P-1.

The spots of saikosaponin a in frs. 1, 2 and 3 were small and light colored. The existence of these was also proved by high performance liquid chromatography (HPLC) as shown in Fig. 6. Some peaks were separated and supposed by the retention time.

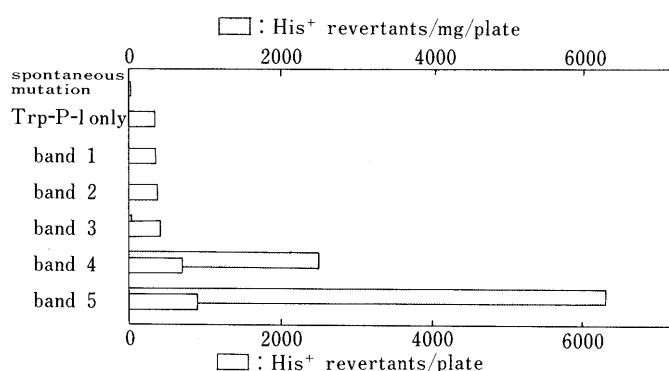


Fig. 7. Effects of Each Band of Fr. 1 Separated by TLC on the Mutagenicity of Trp-P-1

0.2 μg/plate of Trp-P-1 was added for the strain TA98 with S9mix.  $\square$ , the number of revertants by each band were calculated by following the equation: revertant colonies =  $\frac{\text{sample} - (\text{N.C.} + \text{P.C.})}{\text{sample (mg)}}$  N.C., spontaneous mutation; P.C., Trp-P-1 only.  $\square$ , revertants by each band obtained from 3.1 mg of fr. 1.

The existence of saikosaponin c in fr. 2, saikosaponin a and c in fr. 3 and saikosaponin a in fr. 1 was observed. The chart of HPLC for fr. 1 was not shown because its peak was very small.

Fraction 1 had a stronger enhancement effect than saikosaponin a, so, 3.1 mg of fr. 1 was further separated by thin-layer chromatography (TLC). Five fluoresced violet spots were separated.

Revertants in each band separated from 3.1 mg of fr. 1 were shown in Fig. 7. Band 4 and band 5 had an enhancement effect on mutagenicity of Trp-P-1 twice. Their enhancing activities per 1 mg of each band were calculated by the Eq. 1.

Revertants of band 4 were about 2500 colonies per mg and in band 5 were about 6100 colonies per mg, so the existence of the enhancement factor(s) in band 4 and 5 was supposed.

**Comparison of the Enhancement Activity of Saiko Extract and Metabolized Saiko Extract** Boiling-water extract inhibited the mutagenic activity of activated Trp-P-1 according to a dose-response relationship above 0.5 mg/plate as shown in Fig. 8. But 10 mg per plate of metabolized Saiko extract enhanced the mutagenic activity of activated Trp-P-1 twice as shown in Fig. 9. Metabolized Saiko extract did not indicate mutagenicity.

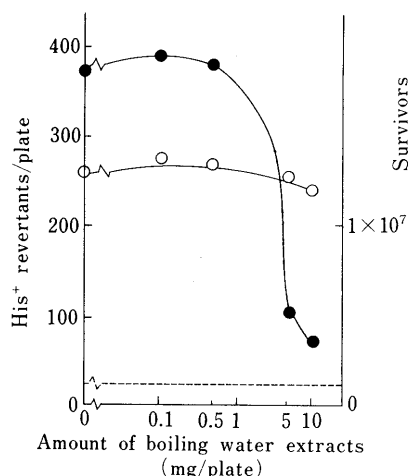


Fig. 8. Effect of Boiling-Water Extract on the Mutagenicity of Activated Trp-P-1

2  $\mu$ g/plate of activated Trp-P-1 was added for the strain TA98 without S9mix. ●—●, revertant colonies; ○—○, survival colonies; ---, spontaneous mutation.

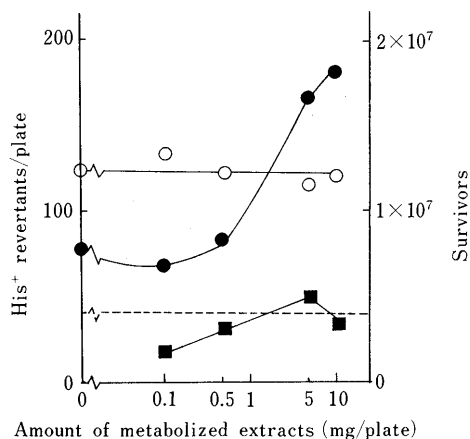


Fig. 9. Effect of Metabolized Extract on the Mutagenicity of Activated Trp-P-1

2  $\mu$ g/plate of activated Trp-P-1 was added for the strain TA98 without S9mix. ●—●, revertant colonies; ○—○, survival colonies; ■—■, revertant colonies of metabolized extracts only; ---, spontaneous mutation.

## Discussion

Some crude drugs have mutagenic activity and some have antimutagenic activity. In this paper an enhancement effect on mutagenicity by Bupleuri Radix was reported.

The enhancement effect on mutagenic activity of mutagen by a crude drug was only reported by Ogawa *et al.* They found the enhancing activity by quercetin on the mutagenicity of 2-acetylaminofluorene and benzo[*a*]pyrene and reported the enhancement effect on mutagenic activity of 2-acetylaminofluorene was due to the inhibition of aryl-hydroxylation in the detoxication pathway.<sup>7)</sup>

In this paper, the effects on mutagenic activity of Trp-P-1 by boiling-water extract from Bupleuri Radix was examined. The mutagenic activity by boiling-water extract was not recognized with or without S9mix but boiling-water extract remarkably enhanced the mutagenic activity of Trp-P-1 with S9mix. Also, boiling-water extract inhibited the mutagenic activity of activated Trp-P-1. Metabolized Saiko extract enhanced the mutagenic activity of activated Trp-P-1, but metabolized Saiko extract does not have mutagenicity. It is supposed that the enhancement activity of Saiko extract

will depend on activation by S9.

The enhancement factor(s) was separated by silica gel chromatography and then TLC. Saikosaponin is well known as a famous substance in Bupleuri Radix. However, the enhancement activity of saikosaponin a was shown to be very weak. The saikosaponin c did not show any activity and saikosaponin d could not be found.

The strongest fraction was fr. 1 and it contained very few saikosaponins. The other components will exist as effective components. Fraction 1 was fractionated to 5 bands and effective components were found in bands 4 and 5. The authors are studying the identification of enhancement factor(s) in bands 4 and 5 of fr. 1. Saikosaponin a is one of the effective components.

## Experimental

**Crude Drugs** Five kinds of Bupleuri Radix were used. Tianjin-Saiko (*Bupleurum chinensis* D.C.), Sho-Saiko (from Kohoku-sho, China, *Bupleurum tenue* BUCH.-HAM. *ex* D. DON), Korean-Saiko (from South Korea, *Bupleurum falcatum* L.) and Japan-Saiko A (from Kumamoto-prefecture, Japan, *Bupleurum falcatum* L.) were purchased from Tochimoto-tenkai-do (Japan). Japan-Saiko B (*Bupleurum falcatum* L.) was collected in the herbal garden of Gifu Pharmaceutical University in October, 1986.

**Chemicals** Benzo[*a*]pyrene was purchased from Nakarai Chemical Co., Ltd., Tokyo (Japan). Trp-P-1 (acetate form) and Trp-P-2 (acetate form) were purchased from Wako Pure Co., Ltd., Osaka (Japan).

*p*-Dimethylaminobenzaldehyde was purchased from Nakarai Chemical Co., Ltd., Tokyo (Japan).

Saikosaponins a, c and d were purchased from Wako Pure Co., Ltd., Osaka (Japan).

**Apparatus** HPLC, Hitachi 655A-12 equipped with a Wakogel-column.

**Preparation of the Saiko Extract** About 100 g of Bupleuri Radix was cut into pieces and put into 1 l of water, immersed for 30 min, and, after boiling for 60 min, the solution was filtrated through a filter paper (Toyo filter paper No. 2) with an aspirator. This procedure was repeated twice. The filtrate was evaporated to dryness under vacuum at 45 °C and the boiling-water extract was obtained. The water content of the extract was measured by reducing weights after the sample was heated in the oven at 80 °C for 8 h. This boiling-water extract was dissolved in 0.1 M phosphate buffer (pH 7.4) and autoclaved at 121 °C for 20 min.

**Preparation of Activated Trp-P-1** According to the method of Arimoto *et al.*,<sup>9)</sup> activated Trp-P-1 was prepared. 0.2 ml of Trp-P-1 aqueous solution (1 g/ml) was mixed to 10 ml of S9mix containing 2 ml of S9 and was incubated at 37 °C for 30 min with shaking. Then acetone of 12 ml was added and the mixture was allowed to stand in an ice-bath for 20 min. After the mixture was centrifuged at 4200  $\times$  *g* for 15 min at 4 °C, the supernatant was collected and evaporated to dryness. The residue, which contained the activated Trp-P-1, was dissolved in water and was subjected to the Ames assay.

**Preparation of Metabolized Saiko Extract** One ml of an aqueous solution of Saiko extract (200 mg/ml) was mixed with 9 ml of S9mix. After the mixture was incubated at 37 °C for 30 min with shaking, the mixture was heated at 60 °C for 15 min for inactivation of the enzyme. This metabolized extract was diluted in 0.1 M phosphate buffer (pH 7.4) and was subjected to the Ames assay.

**Bacterial Assay for Enhancement Effect on Mutagen *Salmonella typhimurium* TA98 and TA100 with S9mix by preincubation method** was used to examine the enhancement activity of Bupleuri Radix extract on mutagen. The S9 fraction was prepared from the liver of Sprague-Dawley rats treated with PCB (Aroclor 1254). 0.5  $\mu$ g Trp-P-1 and 0.2  $\mu$ g Trp-P-2 were dissolved in 0.1 ml of sterilized distilled water, respectively. Benzo[*a*]pyrene of 5  $\mu$ g was dissolved in 0.1 ml dimethylsulfoxide (DMSO).

One tenth ml of the boiling-water extract, 0.5 ml of S9mix, 0.1 ml of mutagen and 0.1 ml of bacterial culture were mixed and preincubated at 37 °C for 20 min. 3 ml of soft agar was added to the mixture and poured onto minimal agar plates. The plates were incubated at 37 °C for 2 d. For the determination of surviving cells, culture dilution of 5  $\times$  10<sup>4</sup> for TA98 and 4  $\times$  10<sup>4</sup> for TA100 were mixed in B-2 agar plates and incubated at 37 °C for 1 d. The numbers of induced revertants and surviving cell colonies were then scored and the frequency of revertants per survivors was calculated. Three plates were prepared for the same sample and the mean

was calculated.

**Separation of Enhancement Substances** Eight hundred and sixty ml of boiling-water extract, containing 22.68 g (dry weight of 15.12 g) of Saiko extract prepared from 100 g of Bupleuri Radix, was fractionated with ether (800 ml) and then *n*-BuOH (800 ml), stepwise. Both extractions were repeated two times. Ether, *n*-BuOH and water layers were evaporated to dryness and 350.5 mg, 2.93 g and 17.84 g of residues were obtained, respectively. Residues of ether and *n*-BuOH layers were dissolved in DMSO and the water layer was dissolved in 0.1 M phosphate buffer. These solutions were pipetted into a petri dish corresponding to 0.5, 5, 25 and 50 mg of Saiko extract, and subjected to the Ames assay. 1/2 the amount of the weight described as before of the residue of the *n*-BuOH layer was used and subjected to the Ames assay. The *n*-BuOH layer (1.8247 g) was chromatographed on silica gel column: (15 mm i.d. × 180 mm) and eluted successively with CHCl<sub>3</sub> (250 ml, fr. 1), CHCl<sub>3</sub>-EtOH (9:1) (700 ml, fr. 2), CHCl<sub>3</sub>-EtOH (7:3) (365 ml, fr. 3), CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (6:4:0.4) (400 ml, fr. 4), CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (4:6:0.5) (100 ml, fr. 5), MeOH (200 ml, fr. 6) and H<sub>2</sub>O (300 ml, fr. 7). Each eluate was evaporated to dryness and obtained 61.3, 31.75, 665.7, 189.5, 40.9, 64.5 and 169.0 mg, respectively. The activities of these residues were tested with Ames assay. Each fraction was further separated by TLC on pre-coated plates of Kieselgel 60 F<sub>254</sub> (Merck). The solvent system for development was ethylacetate-EtOH-H<sub>2</sub>O (18:2:1).

From fr. 1 (54.6 mg) five bands were detected on the plate by exposure to ultraviolet light of wavelength 254 nm.

Bands 1, 2, 3 and 5 fluoresced a violet color and band 4 fluoresced a light-blue color.

Each band was scraped off and extracted with ethylacetate. After evaporated to dryness under vacuum the amounts of 6.0, 4.1, 9.3, 2.1 and 1.5 mg were obtained, respectively. The concentrates were subjected to Ames assay and the effective band was detected.

**TLC Analysis of Saikosaponins in *n*-BuOH Layer** Saikosaponins are well known components in Bupleuri Radix and so their existence in each fraction (frs. 1—7) was examined by TLC.

Spots of saikosaponins on thin-layer plates were detected as pink spots by 1% ethanol solution of *p*-dimethylaminobenzaldehyde and 50% ethanol solution of sulfuric acid followed by heat treatment.<sup>10)</sup> Each spot of saikosaponins was compared with the *R<sub>f</sub>* values of saikosaponins a, c and d.

**HPLC Analysis** The existence of saikosaponins was also proved by HPLC analysis.

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