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Study on Ginseng Protopanaxadiol and Protopanaxatriol Saponins-Induced Antinociception

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We studied the effects of ginseng protopanaxadiol (PD) and protopanaxatriol (PT) saponins on the analgesia using several pain tests such as writhing, formalin, and tail-flick test. Using mouse, pretreatment of PD or PT saponins (i.p.) induced inhibition of abdominal constrictions caused by 0.9% acetic acid administration(i.p.). The AD_{50} was around 27 (17-43) mg/kg for PD and 13.5 (3-61) mg/kg for PT saponins in writhing test. Both PD and PT saponins also showed the inhibition of bitings and lickings of hindpaw after administration of 1% formalin. In particular, both PD and PT saponins showed analgesic effects on second phase of pain. The AD_{50} was 44.5 (26-76) mg/kg for PD and 105 (55-200) mg/kg for PT saponins in second phase of formalin test. For first phase pain inhibition by PD or PT saponins, they were required higher concentrations. However, PD saponins showed weak analgesic effects in tail-flick test with high concentration. In conclusion, we found that both PD and PT saponins have the analgesic effects in writhing test and second phase of pain in formalin test. These results suggest that both PD and PT saponins inhibit neurogenic or tonic pain rather than acute pain.

Key Words: Pain, Ginseng PD and PT saponins, Differential antinociception

INTRODUCTION

Ginseng is the root of Panax ginseng C. A. Mayer (Araliaceae), a well-known oriental folk medicine from long time ago and is used by far east, south east countries, Europe and even Russia. In north america, ginseng is also recently cultivated and is now in markets for keeping one healthy or naturopathic treatment. Ginseng is one of the prototypical herbal medicines consumed in all around world.

Ginseng saponins or ginsenosides isolated from ginseng are main pharmacoactive molecules of ginseng (Kaku et al, 1975). Ginseng saponins show a variety of efficacies such as anticancer, antihypertension, antidiabetes, antistress, antinociception, facilitating learning, and improving the weak body conditions as tonics (Lie & Xiao, 1992). Although ginseng or ginseng saponins are thus used for multiple purposes, it is not proved exactly for its therapeutical efficacy. The cellular or molecular mechanism of ginseng action is not even known.

We demonstrated recently that ginseng root extract or ginsenoside Rf inhibits N-type (and other high-threshold) Ca^{2+} channels in rat sensory neurons with dose-dependent manner (Nah & McCleskey, 1994; Nah, et al, 1995). The inhibitory effect of ginseng extract or ginsenoside Rf on Ca^{2+} channel activity is mediated via a pertussis toxin-sensitive GTP-binding protein(s). Interestingly, we observed that a maximal dose of ginsenoside Rf inhibits Ca^{2+} channels current in sensory neurons to the same extent (> 20%) as maximal activation of the μ-opioid receptor by its selective agonist, DAMGO. The inhibition of Ca^{2+}-evoked neurotransmitter release from sensory neurons is known to be a key element in opioid pain inhibition in the spinal cord, and the ability of ginsenoside

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Rf to block N-type Ca^{2+} channels to the same extent as opioid is strongly predictive of an antinociceptive action of this ginsenoside. In particular, these results support the previous reports that ginseng saponins have antinociceptive action. However, previous reports performed analgesic experiments using only total ginseng saponins (Nabata et al, 1973) and did not characterize the analgesic effects of ginseng saponins.

The aim of this study is to investigate the influence of ginseng saponins, particularly protopanaxadiol (PD) or protopanaxatriol (PT) saponins, relieving the pain induced by various ways such as chemicals or thermal stimulation. If they have the analgesic activity, we will compare the analgesic potency between PD and PT saponins because major ginsenosides classified as PD saponins not only show a slight inhibition of Ca^{2+} channels in rat sensory neurons (Nah, et al, 1995) but also PD and PT saponins differ from physiological and pharmacological properties (Kaku et al, 1975). We will also compare the effects of PD or PT saponins with morphine on antinociception, respectively.

We found that both PD and PT saponins have the analgesic effects in writhing test and second phase of pain in formalin test. These results suggest that both PD and PT saponins inhibit neurogenic or tonic pain rather than acute pain.

METHODS

Animals

ICR mouse (20~30 g) was used for analgesic experiments. eight to ten mice were used for each point of data.

Chemicals

PD and PT isolated from red ginseng root were provided from Korea Ginseng and Tobacco Research Institute. Morphine, and other agents were obtained from Sigma (St. Louis, MO). Ginseng saponins and other drugs are dissolved in saline.

Analgesic Experiments

Writhing test: Koster et al. (1959) for the first described abdominal constrictions produced by intraperitoneal injection of dilute acetic acid in mice. Using this method, mice were placed in the plastic observation box (14×20×25 cm) and allowed to habituate to this novel environment for 60 min. They were then weighed, injected with the test substance intraperitoneally and returned to the box. Thirty minutes later, 0.9% solution of acetic acid was injected i.p. in a volume of 10 ml/kg. Immediately, the number of abdominal constrictions (stretching or extension of hind limbs with a concomitant concave arching of the back) were counted and recorded during 5 min blocks. Three mice were observed simultaneously by a single experimenter.

Formalin test

A slightly modified version of the technique of Hunskaar and his colleagues was used with mice (1985) [originally described by Dubuisson and Dennis (1977) in rats and cats]. 1% formalin was prepared from the aqueous solution of 37% w/w formaldehyde. In this assay, mice were introduced to the testing environment, i.e., 30 cm high, 20 cm diameter plexiglass box for 60 min before any injection. A mirror was placed behind the cylinders for easy observation of whole body of testing animal. They were then weighed and returned to the cylinders. After twenty minutes, i.p. injection of the test substance, 40 μl of 1% formalin was injected just under the skin of the plantar surface of the left hindpaw by use of a microsyringe with a 29-gauge needle. Mice were returned to the cylinders and immediately observed for bitings and lickings of the affected hindpaw. The total time that spent bitings and lickings the left hindpaw over the next 40 min was measured with a stopwatch and recorded to the nearest second in 5 min blocks during both phases as an indicator of nociception. Based on pilot data and in keeping with the literature, the first phase was defined as 0 to 10 min post-injection of formalin and the second phase as 11 min to 40 min post-injection.

Tail-flick test

The tail-flick assay was performed according to the method of D'Amour and Smith (1941) using mice. The intensity of the heat was set to give a baseline reaction latency time of 3-5 s. An automatic cut-off

time of 15 s was used to prevent tissue damage. For the measurement of the basal latency time of the tail-flick response, mice were gently held with the tail positioned in the apparatus (IITC Life Science, USA, Model 33 tail-flick analgesy meter) for radiant heat stimulation. Mice were treated with test substance by i.p. Then, the tail-flick response after treated of ginseng saponins was measured for 30, 60, 90, 120, and 240 min as did for basal latency time.

Wilcoxon Rank Sums test (Mann-Whitney U test) subsequent to nonparametric statistical analysis was used for the nociceptive tests. Values of $p<0.05$ in the two-tailed tests were taken to represent significance. In the AC and FT tests, antinociception was defined as a decrease of nociceptive scores compared to vehicle-treated controls. Antinociception was expressed as percent antinociception calculated as follows;

$$\% \text{ antinociception} = \frac{\text{Mean no. of constriction/ time spent bitings \& lickings by control group} - \text{mean no. of constrictions/ time spent bitings \& lickings by drug-treated group}}{\text{Mean no. of constriction/ time spent bitings \& lickings by control group}} \times 100$$

These values were then used to generate dose-response curves (DRCs). The DRCs were analyzed for slope and interpolated AD_{50} by linear regression of probit-transformed percent analgesia scores.

Only scores between 15% and 85% were used in the analysis to avoid extreme value bias by Litchfield and Wilcoxon (1949).

RESULTS

We did study the analgesic activity of PD or PT saponins prepared from ginseng total saponins using writhing test. As shown in Figs. 1 and 2, vehicle (control) group did show the typical pattern of writhings after administration of 0.9% acetic acid. The pain behavior (writhing) appeared to reach a peak on 5-15 min and disappeared 25-30 min later. However, pretreatment with PD or PT saponins attenuated writhings induced by 0.9% acetic acid. The effect of PD saponins was dose-dependent manner in the range

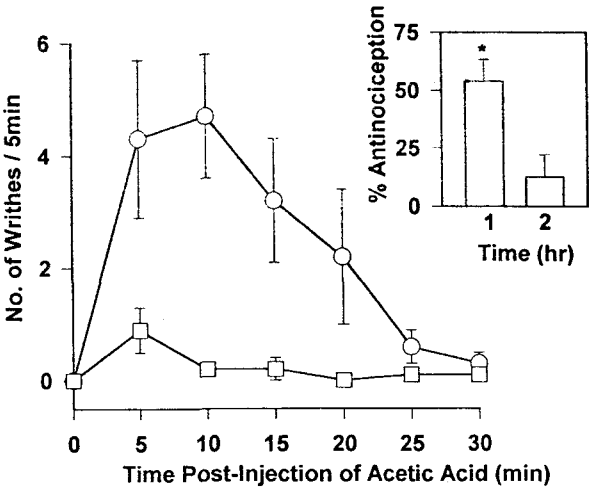

Fig. 1. PD saponins induced-analgesia.
Saline (○) or PD (□) 75 mg/kg was injected with i.p. administration for 30min. After 30 min, 0.9% acetic acid was injected with the same route and then the number of writhing was counted for 30 min with 5 min block. *Inset*; Saline or PD 75 mg/kg was injected with i.p. administration for 1 hour or 2 hours. After 1 hour or 2 hours, 0.9% acetic acid was injected with the same route and then the number of writhing was counted for 30 min with 5 min block. *p<0.01 compared to control (by Student *t* test with unpaired in the two-tailed test).

of 25～100 mg/kg. The effect of PT saponins also was dose-dependent in the range of 12.5～100 mg/kg. The AD_{50} was about 27 (17～43) mg/kg for PD and 13.5 (3～61) mg/kg for PT saponins (Fig. 3 and Table 1). In this test, PT saponins thus have a little lower AD_{50} than PD saponins. The duration of analgesic effect after the administration of PD saponins (75 mg/kg i.p.) maintains for only one hour (Fig. 1, *inset*). But treatment of PT saponins (50 mg/kg) showed the longer duration of analgesic effect than PD saponins (Fig. 2, *inset*).

We used formalin to characterize further analgesic activity of PD or PT saponins, since formalin test is known to have two kinds of pain phase. As shown in Fig. 4 and 5, administration of 1% formalin onto intraplantar surface of hindpaw immediately induced pain expressed as lickings or bitings of affected area. The pain continues for 0 - 10 min (first phase) and disappeared for several minutes. After this period, mouse again starts to lickings or bitings for 10 - 40 min (second phase) after formalin. In our test using PD or PT saponins, less than 200 mg/kg of PD or

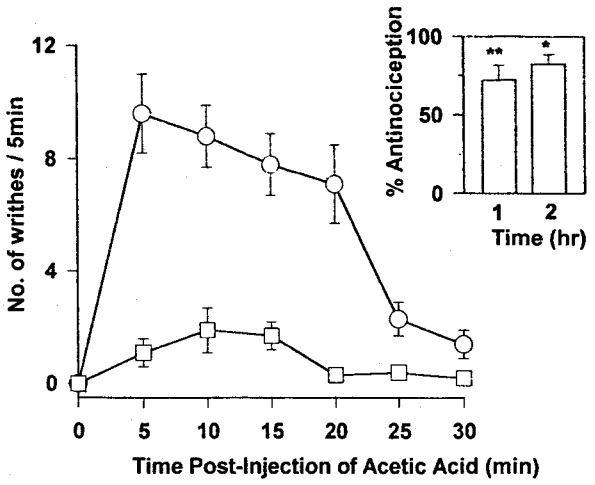

Fig. 2. PT saponins induced-analgesia.
Saline (○) or PT (□) 50 mg/kg was injected with i.p. administration for 30min. After 30 min, 0.9% acetic acid was injected with the same route and then the number of writhing was counted for 30 min with 5 min block. *Inset*; Saline or PT 50 mg/kg was injected with i.p. administration for 1 hour or 2 hours. After 1 hour or 2 hours, 0.9% acetic acid was injected with the same route and then the number of writhing was counted for 30 min with 5 min block. *p<0.01, **p<0.001 compared to control (by Wilcoxon rank sums test subsequent to nonparametric method).

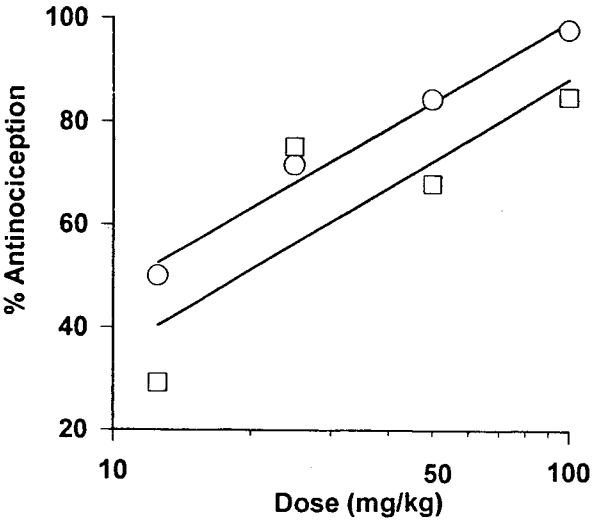

Fig. 3. Dose-dependent effects of both PD and PT saponin on pain.
Saline (○), PD (□), or PT (△) saponins was administrated with indicated dose. After 30 min, writhings are induced by i.p. injection of 0.9% acetic acid and are counted for 30 min. The number of mouse used for experiments is 8-10. Error bars were omitted in the dose response curve for sake of clarity.

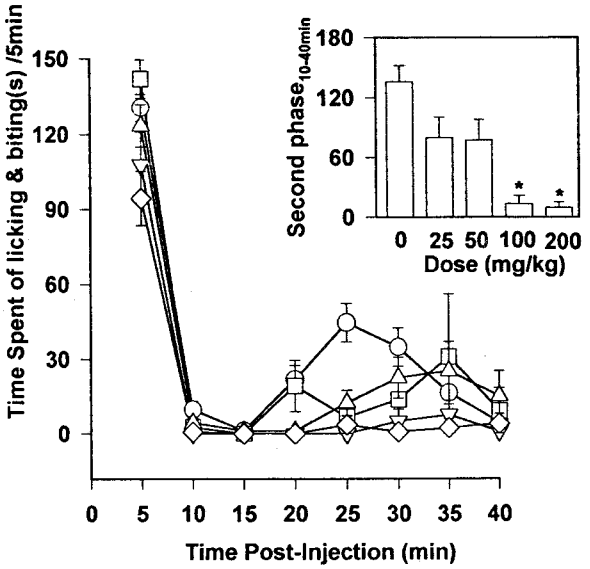

Fig. 4. The effect of PD saponins on pain induced by 1% formalin.
Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of PD saponins. Pain responses were measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain responses are the time that spent of licking and biting(s) of the injected hind paw or leg. Each value represents the mean±SEM. *Inset*; this histograms show only the second phase during 10-40 min following the injection of formalin after pretreatment with different doses of PD saponins. *p < 0.001 compared to saline- injected controls (by Wilcoxon rank sums test subsequent to nonparametric method).

PT saponins failed to show any inhibition of first phase of pain. The administration of only 200 mg/kg before formalin only slightly diminished the pain behavior (data not shown) such as biting or licking of hind paw during first phase of pain, respectively. However, the same amount of PD and PT saponins clearly reduced pain behavior during second phase of pain as shown in Fig. 4 and 5. Interestingly, PD saponins of 100 mg/kg also inhibited the second phase pain. The AD_{50} was 44.5 (26~76) mg/kg for PD and 105 (55~200) mg/kg for PT saponins in second phase of formalin test. In this test, PD saponins thus have a little lower AD_{50} than PT saponins (Table 1).

An acute pain is usually induced by noxious thermal stimulation. We used tail-flick test to confirm the analgesic efficacy on tonic pain from formalin test. We also tested the effect of morphine for comparision

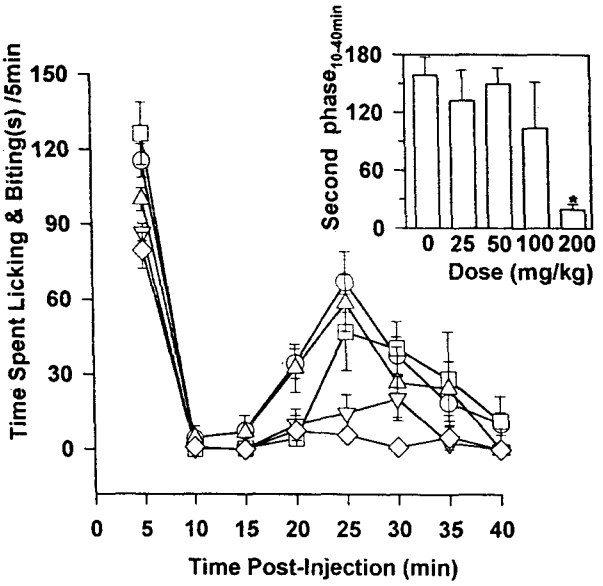

Fig. 5. The effect of PT saponins on pain induced by 1% formalin.
Con (○), 25 (□), 50 (△), 100 (▽), or 200 (◇) mg/kg of PT saponins. Pain response was measured from immediately with 5 min block after intraplantar surface injection of 40 μl of 1% formalin. Pain response is the time that spent of licking and biting(s) of the injected hind paw or leg. Each value represents the mean±SEM. *Inset*; this histograms show only the second phase during 10-40 min following the injection of formalin after pretreatment with different doses of PT saponins. *p < 0.001 when compared to saline-injected controls (by Wilcoxon rank sums test subsequent to nonparametric method).

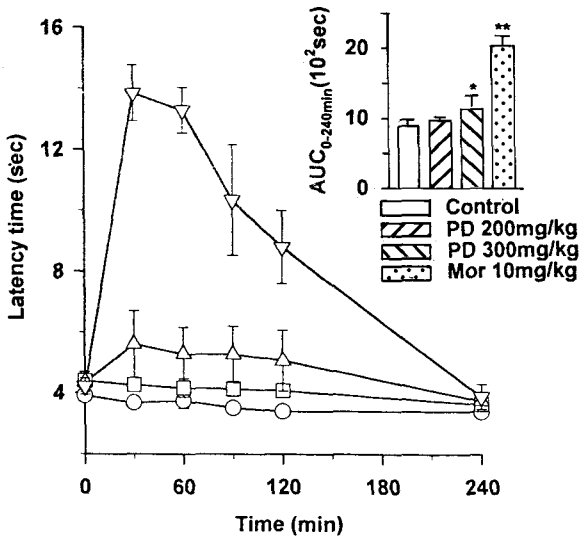

Fig. 6. The effect of PD saponins on noxious heat stimulus.
Con (○), 200 (□), 300 (△) mg/kg of PD or morphine (▽) 10 mg/kg. Animals responded to a focused-heat-stimulus by flicking or removing their tail. The reation time was recorded for control and for pretreated with PD or morphine. This figure also shows time course of antinociceptive effects of pretreatment with saline, PD or morphine on tail-flick latency. Tail-flick latency time was measured after 0, 30, 60, 90, 120, and 240 min following pretreatment with PD saponins or mophine. *Inset*; The analgesic response was transformed into tail-flick latency time (or response) into area under curve (AUC)±SEM. PD (300 mg/kg) or morphine pretreated animals are significantly different compared to control group. *p< 0.05, **p<0.001 (by Wilcoxon rank sums test subsequent to nonparametric method)

Table 1. Analgesic effect of PD or PT saponins on writhing and formalin test

Ginseng saponins (mg/kg)	Writhing test	Second phase of formalin test
	AD_{50}(Lower limit/Upper limit)	
PD	27(17/43)	44.5(26/76)
PT	13.5(3/61)	105(55/200)

with PD or PT saponins at the same time. As shown in Fig. 6 (*inset*), the administration of PD saponins (200 mg/kg) did not show any analgesic efficacy but high concentration of PD saponins (300 mg/kg) was required to get a weak analgesic effects. In experiment using PT saponins, the administration of PT saponins (200~400 mg/kg) did not show any analgesic effects (Fig. 7). In contrast, the administration of morphine (10 mg/kg) showed strong analgesic activity from 30 min to near 4 hours (Figs. 6 and 7).

DISCUSSION

The degree of analgesic activity or analgesic efficacy of certain drugs was usually proved or estimated by using at least three independent testing methods such as writhing, formalin, and tail-flick test (Koster et al, 1956; Hunskaar et al, 1985; Dubuissin & Dennis, 1966; D'Amour & Smith, 1941). To test

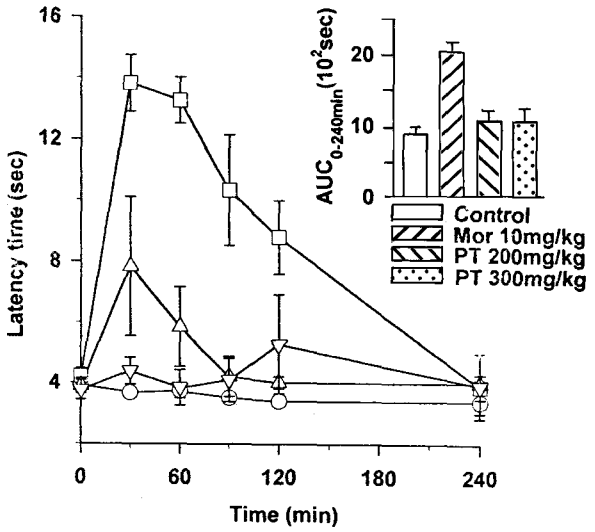

Fig. 7. The effect of PT saponins on noxious heat stimulus.
Con (○), 200 (▽), 300 (△) mg/kg of PT or morphine (□) 10 mg/kg. Animals responded to a focused-heat-stimulus by flicking or removing their tail. The reation time was recorded for control and for pretreated with PT or morphine. This figure also shows time course of antinociceptive effects of pretreatment with saline, PT or morphine on tail-flick latency. Tail-flick latency time was measured after 0, 30, 60, 90, 120, and 240 min following pretreatment with PT saponins or mophine. *Inset*; The analgesic response was transformed into tail-flick latency time (or response) into area under curve (AUC) ± SEM. Morphine pretreated animals are significantly different compared to control group. *$p<0.001$ (by Wilcoxon rank sums test subsequent to nonparametric method)

the analgesic effects of PD or PT saponins, we first performed the writhing test, since this test is easy to check and pain is induced by just injection of dilute acetic acid. Using this method, we found that both PD and PT saponins have analgesic effects with dose dependent manner as reports that used ginseng total saponins (Nabata et al, 1973). In addition, we found that PT saponins had a slight higher potency for induction of analgesia than that of PD saponins (Fig. 3). The duration of analgesia induced by PT saponins appeared also longer than PD saponins. Interestingly, the dose (50 mg/kg) of PT saponins induces the analgesia for two hours but the analgesic effects by the same dose of PD saponins only persist 30 minutes (data not shown). The analgesic effects of PD saponins maintained one hour at even 75 mg/kg of PD saponins (Figs. 1 and 2). This difference in analgesic duration of PD or PT saponins remain to be explained.

We also need to further characterize the analgesic activity of PD or PT saponins, since previous reports did not show the evidences that ginseng saponins have an efficacy on what kind of pains (Nabata et al, 1973; Ramaro & Bhargava, 1990). For this purpose, we tested the effects of PD or PT saponins on formalin-induced pain. The formalin test induces two phases of pain by local injection of low percent of formalin (Shibata et al, 1990). The first phase of pain is induced for 0-10 min after formalin administration and this pain is due to the direct stimulation of nociceptor in peripheral nerve by formalin (Hunskaar et al, 1985; Dubuissin & Dennis, 1966; Shibata et al, 1990). The second phase of pain is induced for 10-40 min after formalin administration. This pain is due to inflammation of peripheral tissue by formalin or due to neural plasticity of central nervous system (Shibata et al, 1990; Tjølsen et al, 1992). Both PD and PT saponins did not show an analgesic effects during first phase of pain. However, PD saponins showed the analgesic effect at the concentrations of 100~200 mg/kg on second phase of pain. PT saponins also showed the analgesic effect at the concentrations of 200 mg/kgon second phase of pain. This result suggests that both saponins have an analgesic efficacy on second phase of pain. We also found that PD saponins had a slight higher potency for induction of analgesia than PT saponins (Table 1). Interestingly, the dose of PD or PT saponins that we used to get analgesic efficacy of second phase of pain is almost same or even a little lower than that of aspirin (Tjølsen et al, 1992).

We have performed another test called tail-flick test to confirm the efficacy that PD and PT saponins have shown on second phase of pain. This test uses a noxious thermal stimulation for induction of pain. Thermal stimulation causes an acute pain (D'Amour & Smith, 1941). Previous reports using this test show that ginseng total saponins have a weak analgesic effects (Ramaro & Bhargava, 1990). However, in our experiments as shown in Fig. 7, PT saponins did not show any analgesic activity on this test with even high concentrations of saponins (300 mg/kg). But morphine prolonged the response time against thermal stimulation and the analgesic effect of morphine persisted near to four hours after morphine adminis-

tration. These results suggest clearly that the analgesic effects or analgesic pathway induced by both saponins are different from those of morphine.

In summary, we found using above mentioned three methods that both PD and PT saponins had the analgesic efficacy on writhing test and second phase of formalin test but faild to show analgesic effect on first phase of formalin test and tail-flick test. These results suggest that PD or PT saponins relieve the tonic pain but not acute pain, although the mechanism of analgesia induced by both saponins is not yet known and is under the further investigation.

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