Original 和漢医薬学会誌6, 58－63, 1989

The effect of Gomi-gan on trypsin-like protease of the mouse submaxillary gland

Aiping HUANG and Zen-ichi OGITA*

Departement of Pathogenic Biochemistry, Research Institute for Wakan-Yaku, Toyama Medical and Pharmaceutical University

(Received November 28, 1988. Accepted March 7, 1989.)

Abstract

This study is mainly focused on the effect of Gomi-gan and of the five components of Gomi-gan, *i.e.*, Corni Fructus, Moutan Radicis Cortex, Poria, Batatatis Rhizoma, Alismatis Rhizoma, on trypsin-like protease activity of the submaxillary gland in both B10A and B10 strain mice. Similar to the effect of Hachimi-jio-gan, Rokumi-jio-gan and Rehmanniae Radix, Gomi-gan also increased trypsin-like protease activity of the submaxillary gland in male, but not in female and castrated male mice, indicating that Gomi-gan also had an androgen-dependent effect on the enzyme. Two the *orbis renalis* (腎)**-tonifying herbs in Gomi-gan, Corni Fructus and Moutan Radicis Cortex, had an androgen-dependent effect on trypsin-like protease of the mouse submaxillary gland, whereas the remaining three herbs, Poria, Batatatis Rhizoma, and Alismatis Rhizoma, which had no direct effect of tonifying the *orbis renalis* had no effect on trypsin-like protease. These data as well as our previous findings indicate that trypsin-like protease activity of the mouse submaxillary gland may be related to the *orbis renalis* described in Oriental medicine. Based on these observations, we suggest that trypsin-like protease activity of the mouse submaxillary gland in males may be used as a model for monitoring the *orbis renalis*-tonifying effect.

Key words Gomi-gan, Corni Fructus, Moutan Radicis Cortex, trypsin-like protease, androgen-dependent effect, mouse, submaxillary gland.

Abbreviations BAPNA, *N*α-benzoyl-D,L-arginine-*p*-nitroanilide ; Gomi-gan (Wu-Wei-Wan), 五味丸 ; Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan), 八味地黄丸 ; Rokumi-jio-gan (Liu-Wei-Di-Huang-Wan), 六味地黄丸.

Introduction

Gomi-gan (Wu-Wei-Wan), consisting of five herbs, Corni Fructus, Moutan Radicis Cortex, Poria, Batatatis Rhizoma and Alismatis Rhizoma is a major part of both Hachimi-jio-gan (Ba-Wei-Di-Huang-Wan) and Rokumi-jio-gan (Liu-Wei-Di-Huang-Wan). Rokumi-jio-gan contains one more herb, Rehmanniae Radix and Hachimi-jio-gan contains three more herbs, Rehmanniae Radix, Aconiti Tuber and Cinnamomi Cortex. These three recipes are invigorators for the syndrome of the *orbis renalls exhausted* (腎虚). With more tonic herbs, Hachimi-jio-gan and Rokumi-jio-gan are more powerful in tonifying the *orbis renalis* than Gomi-gan, and Hachimi-jio-gan supports the *orbis renalis*-yang as well, according to the description in Oriental medicine.

In the previous studies, Taie *et al.*[1-3] have shown that Hachimi-jio-gan and Rokumi-jio-gan increase trypsin-like protease activity of the mouse submaxillary gland in both B10A and B10 male mice. They have no effect on the enzyme found in female and castrated male mice of both strains, with the exception that Hachimi-jio-gan

*〒930-01 富山市杉谷2630 富山医科薬科大学 和漢薬研究所病態生化学部門 荻田善一 2630, Sugitani, Toyama 930-01, Japan

**The *orbis renalis* (腎) is a technical term in the Chinese medicine.

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **6**, 58－63, 1989

has some effect on trypsin-like protease of female and castrated male in B10A mice. For a single herb effect, Taie *et al.* have shown that Aconiti Tuber extract as well as aconitine, a major element of Aconiti Tuber, also increases trypsin-like protease activity of the mouse submaxillary gland in both B10A and B10 male but not female and castrated male mice. No effect of Cinnamomi Cortex as well as cinnamaldehyde, a major element of Cinnamomi Cortex is observed on the enzyme of both strains in male, female and castrated male mice. We have shown[4] that Rehmanniae Radix increases the enzyme in male, but not in female and castrated male mice in both the B10A and B10 strains. This Rehmanniae Radix effect on trypsin-like protease can be induced in castrated male mice when both Rehmanniae Radix and testosterone are administered. In continuing the study, the present experiment is designed to further test the effect of Gomi-gan and the five herbs in Gomi-gan on trypsin-like protease of the submaxillary gland in both strain of mice.

Materials and Methods

Materials : Batatatis Rhizoma, *Dioscorea batatas* DECAISNE, produced in Henan (河南) province of China, Alismatis Rhizoma, *Alisma plantago-aquatica* L. subsp. *Orientale* SAMUELSSON, produced in Shichuan (四川) province of China, Poria, *Poria cocos* (FR.) WOLF *pachyma hoelen* RUMPH, produced in Guizhou (貴州) province of China, Moutan Radicis Cortex, *Paeonia moutan* SIMS, produced in Anhui (安徽) province of China, Corni Fructus, *Cornus officinalis* SIEB. et ZUCC, produced in Korea.

Animals : Eight-week-old B10A and B10 strain mice, male and female, were used in this experiment. The animals were kept in rooms with controlled temperature (23±2℃) and humidity (55±10%) and supplied with standard mice chow and water *ad libitum*. Castration of the male mice was carried out under nembutal anesthesia (0.01 mg/g B.W.) at the age of 4 weeks and kept for another 4 weeks before use.

Extraction of the herbs : Ten percent (W/V) of each herb in 99% ethyl alcohol was heated for 3 hr at the temperature of 55℃. The extract, filtered by passage through cotton, was evaporated by mild heating in a vacuum and continuous stirring until a dense syrup was obtained. The syrup 1.0, 4.0, 8.0 (Gomi-gan, 五味丸) ; 4.6 (Corni Fructus, 山茱萸) : 0.14 (Batatatis Rhizoma, 山薬) ; 0.68 (Alismatis Rhizoma, 沢瀉) ; 0.12 (Poria, 茯苓) ; 1.76 (Moutan Radicis Cortex, 牡丹皮) g/kg B.W./day respectively was suspended in water and was administered orally to each animal by means of a metal gastric tube at 2 : 00 to 3 : 00 p.m. daily for 14 days. The control mice were administered with distilled water.

Tissue preparation : The mice were sacrificed by cervical dislocation. The submaxillary glands were removed, and the blood was washed away in ice cold saline and dissected free of the adipose and lymphatic tissue. The tissue was homogenized in 9 volumes of deionized water at 4℃ in a glass homogenizer and centrifuged at 16,000 × *g* for 30 min. The supernatants were used immediately for the measurement of enzyme activity, protein content assay and electrophoretic analysis.

Spectrophotometric assay for trypsin like protease activity : Trypsin-like protease activity in submaxillary glands was measured by the methods of Taie and Ogita.[5] The tissue extract (0.5 ml) was incubated at 37℃ for 60 min with 1.0 ml of substrate solution containing 1 mM *Nα*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA), 2% of dimethylsulfoxide (DMSO) and 50 mM phosphate buffer (pH 7.6). The reaction was stopped by addition of 0.5 ml of 20% perchloric acid. The mixture was then centrifuged at 3,000 × *g* for 30 min at room temperature, and 1.0 ml of the supernatant was mixed with 1.0 ml of pre-cooled 0.2% sodium nitrite solution and kept in an ice bath for 10 min. One milliliter of 0.5% ammonium sulfamate solution was added to the solution to destroy the excess sodium nitrite. Two milliliters of 0.05% *N*-1-naphthylethylenediamine dihydrochloride solution was added and incubated at room temperature for 30 min. The absorbance at 546 nm was measured in a Hitachi model 200/20 spectrophotometer. Protein content in the supernatant was measured by the

methods of Lowry *et al.*[6] Bovine serum albumin (Sigma Chemical Co.) was used as a standard. One unit was defined as the amount of enzyme required to hydrolyze 1 μmol of BAPNA in 1 min at 37℃. Specific activity was indicated as one unit of enzyme in 1 mg of total protein.

Statistical analysis : The differences between the control group and the experimental groups were statistically evaluated using the Student's *t*-test.

Results

Gomi-gan had a significant effect of increasing trypsin-like protease activity of the submaxillary gland in B10A and B10 strain male mice. However, the strain related difference is quite obvious. In B10A male mice, trypsin-like protease activity was increased by administration of small (1 g/kg B.W./day), medium (4 g/kg B.W./day) and large doses (8 g/kg B.W./day) of Gomi-gan extract with $p<0.05$, $p<0.01$ and $p<0.002$, respectively, whereas in B10 male mice, the enzyme activity was significantly increased only by a large dose of Gomi-gan extract with $p<0.05$, and was not affected by small and medium doses of Gomi-gan (Fig. 1). For the effect of a single herb in Gomi-gan, Poria (Por), Batatatis Rhizoma (B.R.) and Alismatis Rhizoma (A.R.) did not affect the activity significantly in both B10A and B10 male mice, whereas Moutan Radicis Cortex (M.R.) and Corni Fructus (C.F.) increased the trypsin-like protease activity of the submaxillary gland in both B10A and B10 male mice with $p<0.05$ and $p<0.01$, respectively (Fig. 2). The doses of each herb we used in this study were based on the previous experiment and calculation from the original recipe. Table I is a summary of the study carried out in our laboratory about the effect of the *orbis renalis*-tonifying recipes and

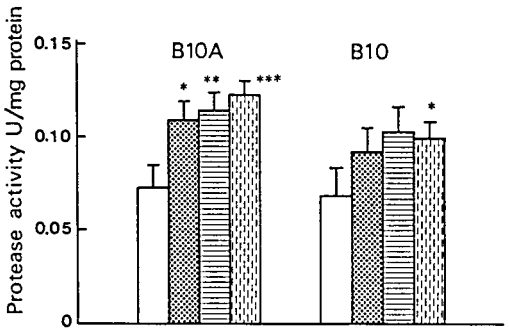

Fig. 1 The effect of Gomi-gan on trypsin-like protease of the submaxillary gland in male of B10A and B10 mice.

Columns represent the mean value for 10 samples and bars indicate S.E. Open column : mice given distilled water as control ; dotted column : mice given Gomi-gan of 1 g/kg B.W./day ; horizontal hatched column : mice given Gomi-gan of 4 g/kg B.W./day ; vertical hatched column : mice given Gomi-gan of 8 g/kg B.W./day. *, **, *** : significantly different from control with $p<0.05$, $p<0.01$, $p<0.002$, respectively.

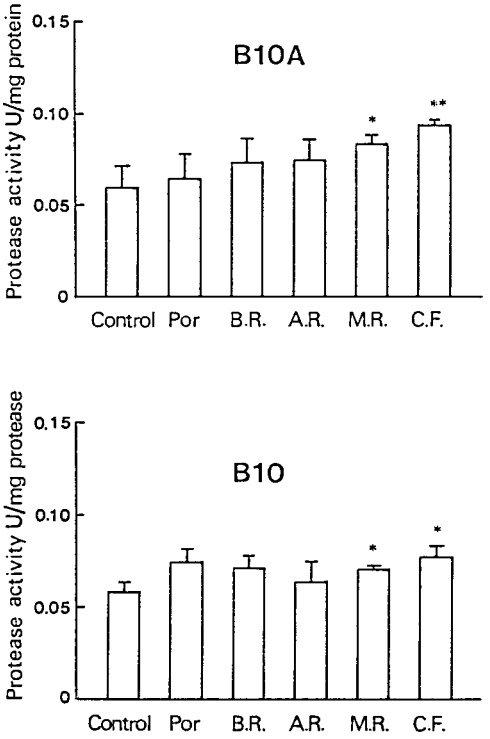

Fig. 2 The effect of each herb in Gomi-gan on trypsin-like protease of the submaxillary gland in male of B10A and B10 strain mice.

Columns represent the mean value for 6 samples and bars indicate S.E. Por (Poria, 茯苓) was given 0.12 g/kg B.W./day ; B.R. (Batatatis Rhizoma, 山薬) was given 0.14 g/kg B.W./day ; A.R. (Alismatis Rhizoma, 沢瀉) was given 0.68 g/kg B.W./day ; M.R. (Moutan Radicis Cortex, 牡丹皮) was given 1.76 g/kg B.W./day ; C.F. (Corni Fructus, 山茱萸) was given 4.6 g/kg B.W./day. *and ** : significantly different from control with $p<0.05$ and $p<0.01$, respectively.

Table I The effect of Oriental medicine on trypsin-like protease in the submaxillary gland of male mice.

Oriental medicines	Strain	
	B10A	B10
Hachimi-jio-gan (八味地黄丸)	↑↑↑	↑
Aconiti Tuber (附子)	↑↑↑	↑
Cinnamomi Cortex (桂枝)	−	−
Rokumi-jio-gan (六味地黄丸)	↑↑↑	↑
Rehmanniae Radix (熟地黄)	↑↑↑	↑
Gomi-gan (五味丸)	↑↑↑	↑
Poria (茯苓)	−	−
Alismatis Rhizoma (沢瀉)	−	−
Batatatis Rhizoma (山薬)	−	−
Moutan Radicis Cortex (牡丹皮)	↑	↑
Corni Fructus (山茱萸)	↑↑	↑

Arrows represent rates of increase in activities of trypsin-like protease after treatment ; ↑↑↑, ↑↑, and ↑ represent 100%−70%, 70%−50% and 50%−20% increments of activities, respectively. No increase in the activity is indicated by −.

herbs on trypsin-like protease activity of the mouse submaxillary gland in B10A and B10 strain mice. From the table, we found out that all the *orbis renalis*-tonifying, Hachimi-jio-gan, Rokumi-jio-gan and Gomi-gan, and single herbs, Aconiti Tuber, Rehmanniae Radix, Moutan Radicis Cortex, Corni Fructus, had an increasing effect of trypsin-like protease activity in male mice, whereas the remaining herbs ; Cinnamomi Cortex, Poria, Alismatis Rhizoma, Batatatis Rhizoma, although they may have some indirect effect of tonifying the *orbis renalis*, had no effect on the enzyme activity. The changes of trypsin-like protease activity in relation to the *orbis renalis*-tonifying effects were more pronounced in B10A strains than in B10 strain mice.

Discussion

Like Rehmanniae Radix, Gomi-gan has a positive effect on trypsin-like protease of the submaxillary gland in male mice but not in female and castrated male mice. The data, again, suggests that the effect of Gomi-gan is androgen-dependent. The effect of Gomi-gan on trypsin-like protease also shows a strain-related difference in cogenic progeny,[7] B10A and B10 mice in a similar pattern to the effect of Rehmanniae Radix, which further indicates the regulatory mechanisms of those drug effects on trypsin-like protease may involve H-2 region on chromosome 17.[4]

In order to further investigate how Gomi-gan effects trypsin-like protease activity, we have analyzed the effect of each single herb in Gomi-gan on trypsin-like protease activity, and found out that only Corni Fructus and Moutan Radicis Cortex have a similar effect to the Gomi-gan's, but the intensity of the drug effect is less than that of Gomi-gan. These suggest that Corni Fructus and Moutan Radicis Cortex are the main components in Gomi-gan in increasing the enzyme activity of the mouse submaxillary gland in males. The remaining three herbs, Poria (Por), Alismatis Rhizoma (A.R.) and Batatatis Rhizoma (B.R.), have no effect *per se* on the activity, but they co-operatively enhance the effect of Corni Fructus and Moutan Radicis Cortex on the activity.

In summarizing all the data on this subject which has been carried out in our laboratory in the past six years, we realize that all the recipes and herbs which have a direct effect of tonifying the *orbis renalis*, including *yin** (陰) and *yang*** (陽), have a positive effect of the trypsin-like protease activity of the submaxillary gland in male mice. The recipes are Hachimi-jio-gan, Rokumi-jio-gan and Gomi-gan, and the herbs are Aconiti Tuber, Rehmanniae Radix, Corni Fructus and Moutan Radicis Cortex. The herbs which have no direct effect of invigorating the *orbis renalis*, Cinnamomi Cortex, Poria, Alismatis Rhizoma, Batatatis Rhizoma, have no direct effect on the enzyme of the submaxillary gland. These suggest that the activity, a sex hormone (androgen) dependent enzyme,[8-10] may be related to the *orbis renalis* (including *yin* and *yang*) which was described in Oriental medicine. This hypothesis matches the other suggestion that the syndrome of *orbis renallas exausted* may involve hypothalamo-hypophysio-gonad dysfunction.[11] Based on the consistency of the tonifying recipes and herbs to the increasing effect of the activity in male mice, we suggest that the activity in males may be a good indication of monitoring the *orbis renalis*-tonifying drug effect. Further study for establishing this model needs to be done. Two things which should be mentioned according to the observations in our study are : first, the changes of trypsin-like protease activity in relation to the effect of tonifying the *orbis renalis* only in male mice, but not in females as well as in castrated male mice, because the trypsin-like protease is an androgen-dependent, that is not what *orbis renalis* means in Oriental medicine. The syndrome of *orbis renallas exausted* is also popular in females and can be improved by administration of *orbis renalis* invigorators of herbs and recipes ; second, the effect on enzyme in males is more pronounced in B10A strain mice and is poorly reflected in B10 strain mice upon administration of *orbis renalis* invigorators, which suggests that B10A strain mice may be a better candidate for establishing the model of monitoring the *orbis renalis*-tonifying.

* *yin* = structive aspect of an effective position.[12]
** *yang* = active aspect of an effective position.

和文抄録

五味丸及びその構成生薬が，B10A 並びに B10 マウスの顎下腺トリプシン様プロテアーゼ活性に及ぼす影響を研究した。六味地黄丸並びに熟地黄と同様に，五味丸にも雄マウスにおいてのみその酵素活性の誘導上昇が認められた。五味丸を構成する５つの生薬エキスを雄マウスにそれぞれ単独に投与したところ，補腎生薬の山茱萸と牡丹皮はトリプシン様プロテアーゼ活性を上昇させたが，他の生薬（山薬，茯苓，沢瀉）にはその酵素活性を上昇させる効果が認められなかった。補腎方剤あるいは補腎生薬においてのみこの酵素活性を上昇させる効果が認められた実験結果から，トリプシン様プロテアーゼ活性は中国医学の「腎」に関与していることが考えられる。補腎方剤及び補腎生薬の効果を研究するために，マウス顎下腺トリプシン様プロテアーゼ活性は１つのよい指標であることが示唆された。

References

1) Taie, H. and Ogita, Z-I. : A significant effect of Hatimi-zio-gan on arginine aminopeptidase in mice submaxillary gland. *J. Med. Pharm. Soc. WAKAN-YAKU* **2**, 411-417, 1985.
2) Taie, H. and Ogita, Z-I. : The effect of Rokumi-zio-gan on arginine aminopeptidase in mice submaxillary gland. *J. Med. Pharm. Soc. WAKAN-YAKU* **3**, 143-147, 1986.
3) Taie, H. and Ogita, Z-I. : Responsiveness of mouse submaxillary gland arginine aminopeptidase to Aconiti Tuber extract. *J. Med. Pharm. Soc. WAKAN-YAKU* **3**, 177-183, 1986.
4) Huang, A. and Ogita, Z-I. : Androgen-dependent effect of Rehmanniae Radix on trypsin-like protease of the mouse submaxillary gland. *J. Med. Pharm. Soc. WAKAN-YAKU* **5**, 191-199, 1988.
5) Taie, H. and Ogita, Z-I. : Colorimetric determination of arginine aminopeptidase in the submaxillary gland of mice. *Japan J. Clin. Chem.* **14**, 405-410, 1985.
6) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. : Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275, 1951.
7) Shreffler, D.C. : Antigenic and immunological loci. In "Genetic variants and strains of the laboratory mouse," Gustav Fischer Verlag, New York, pp. 424-425, 1981.

8) Angeletti, P.U. and Angeletti, R. : Androgen-dependent esterases activity in the mouse submaxillary gland. *Biochim. Biophys. Acta* **136**, 187-190, 1967.

9) Calissano, P. and Angeletti, P.U. : Testosterone effect on the synthetic rate of two esteropeptidases in the mouse submaxillary gland. *Biochim. Biophys. Acta* **156**, 51-58, 1968.

10) Riekkinen P.J. and Niemi, M. : Androgen-dependent salivary gland protease. *Endocrinology* **83**, 1224-1231, 1968.

11) Chunhua, J., Xueli, Z., Tianjue, G. and Ziyin, S. : "Jin no Gendaiigakuteki Kenkyu," Chugokukanpou, pp. 34-47, 179-194, 256-267, 1985 (in Japanese).

12) Porkert, M. : In "The theoretical foundations of Chinese medicine," The MIT Press, Cambridge, Massachusetts, p. 14, 1985.