The effect of Rokumi-gan on arginine aminopeptidase in submaxillary gland of mice

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Abstract

The activity of arginine aminopeptidase in the submaxillary gland was examined in two selected mouse substrains, HR-A and HR-B. Two weeks treatment with Rokumi-gan (Liu-Wei-Wan) in diet (10 % grams/day/mouse) resulted in an increase in the activity of arginine aminopeptidase in the male mice but not in the female or castrated male mice of both substrains. Therefore, testes are involved in the stimulation of arginine aminopeptidase activity after Rokumi-gan treatment.

Key words Oriental medicine "Rokumi-gan" arginine aminopeptidase, mouse submaxillary gland

Abbreviations BAPNA, αN-Benzoyl-DL-arginine-p-nitroanilide ; Hatimi-ziô-gan (Ba-Wei-Di-Huang), 八味地黄丸 ; Rokumi-gan (Liu-Wei-Wan), 六味丸

Introduction

Arginine aminopeptidase is a biochemical marker for the estimation of androgenic action of medicines since it's activity is dependent on testicular androgenic.[1,2] A diet containing Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan) Oriental medicine causes an increase in arginine aminopeptidase activity in mouse submaxillary glands in intact male, female and castrated male mice of two selected substrains. In our study Hatimi-ziô-gan directly acted on the submaxillary gland and increased arginine aminopeptidase activity in HR-A substrain mice. However, in HR-B substrain mice Hatimi-ziô-gan indirectly increased arginine aminopeptidase activity by increasing testicular androgen secretion.[3] Rokumi-gan is an Oriental medicine which contains two less herbs than Hatimi-ziô-gan, Cinnamomi Cortex and Aconiti Tuber, but is otherwise identical to Hatimi-ziô-gan. To determine if action of Rokumi-gan on arginine aminopeptidase activity is direct or indirect, it was added to the diets of intact or castrated male and female mice of both HR-A and HR-B mouse substrains.

Materials and Methods

Chemicals : αN-Benzoyl-DL-arginine-p-nitroanilide HCl (BAPNA) was purchased from Nakarai Chemicals, Ltd. (Tokyo, Japan) ; cellulose acetate membrane (60×220) was purchased from Macherey Nagel Co. (Germany) ; N-1-naphthylethylenediamine dihydrochloride, acrylamide, N,N-methylene bisacrylamide, and other

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reagents used were analytical grade products obtained from Wako Pure Chemical Industries (Tokyo, Japan). Rokumi-gan was obtained from Ohminedo Co., Ltd. (Nara, Japan). Rokumi-gan consists of six components ; Rehmanniae Radix 8.0 g, Corni Fructus 4.0 g, Dioscoreae Rhizoma 3.0 g, Alismatis Rhizoma 3.0 g, Hoelen 3.0 g, Moutan Cortex 3.0 g, in 250 g of mice chow.

Animals and treatment : Experimental animals used in the present study were four week-old of both sexes of HR-A and HR-B substrains of mice. Detailed informations concerning the selection of these substrains were previously described.[3] They were kept under a constant light-dark regimen (dark from 7 : 00 P.M. to 6 : 00 A. M.), constant environmental temperature (23± 2℃) and constant humidity (55±10 %) condition.

Male mice were castrated after being anesthetized with nembutal (0.02 mg/g body weight, i.p.). Four weeks after castration, the animals were devided into two groups. The first group was fed a diet containing 10 % Rokumi-gan for 2 weeks, while the second group was fed a common diet (Rokumi-gan-free) as controls (Clea Japan, Inc.).

Preparation of submaxillary glands extract : The animals were killed by cervical dislocation, and the submaxillary glands were quickly removed and carefully freed from adipose and lymphatic tissue. The glands were washed with ice-cold 0.9 % NaCl solution to remove traces of blood, and were homogenized with 9 volumes of deionized water at 4 ℃. The homogenates were centrifuged at 20000×g for 30 minutes at 4 ℃. The resulting clear supernatants were used for enzymatic assay and electrophoretic analysis.

Assay of enzyme activity : The activity of arginine aminopeptidase was measured by the method of Taie and Ogita[4] using αN-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate. One unit of arginine aminopeptiase activity is defined as the amount of enzyme releasing 1 μM of p-nitroaniline in 1 minute at 37 ℃.

Electrophoretic analysis : Polyacrylamide gel electrophoresis was carried out according to the method of Ogita and Markert.[5] Electrophoresis was performed in a vertical 7 % polyacrylamide gel slab. A constant current of 15 mA was applied for 120 minutes at 4 ℃. The staining of arginine aminopeptidase was accomplished by the methods of Isobe and Ogita.[6] Following electrophoresis, 10 mg of BAPNA substrate was completely dissolved in 0.5 ml of dimethylsulfoxide, and mixed with 2 ml of 0.1 M phosphate buffer (pH 7.6). The cellulose acetate membrane (220× 60 mm) which had been immersed in substrate solution, was placed on the gel and incubated at 37 ℃ until yellow bands of p-nitroaniline appeared. After incubation the membrane was immersed for 15 minutes in a staining solution consisting of 40 ml of 0.1 M Tris-HCl buffer (pH 8.8), containing 50 mg of N-1-naphthylethylenediamine dihydrochloride and 100 mg of sodium nitrite. Then the membrane was rinsed in 40 ml of deionized water containing 500 mg of ammonium sulfamate, and then was immersed in 2 N HCl solution. Arginine aminopeptidase activity was revealed as clear red purple bands on the cellulose acetate membrane.

Results and Discussion

The present results showed that there was an increase in arginine aminopeptidase isozymes of the submaxillary gland in both HR-A and HR-B substrains mice given a diet containing 10 % Rokumi-gan for two weeks (Figs. 1 and 2). Similar results were obtained using spectrophotometric analysis (Fig. 3). However, the increase caused by treatment with a 10 % Rokumi-gan containing diet was not observed in the castrated mice of either HR-A or HR-B substrain (Fig. 4). Moreover, no arginine aminopeptidase activity was demonstrated in, Rokumi-gan-fed, female mice neither in HR-A nor HR-B substrains (Fig. 5). This indicates that Rokumi-gan has no direct effect on arginine aminopeptidase activity in the submaxillary gland. Therefore it is possible that Rokumi-gan stimulates testicular androgens which in turn stimulate arginine aminopeptidase activity in the submaxillary gland. It has been reported that enzymes which accumulate in submaxillary gland are controled by androgens hormones.[7,8] The different pat-

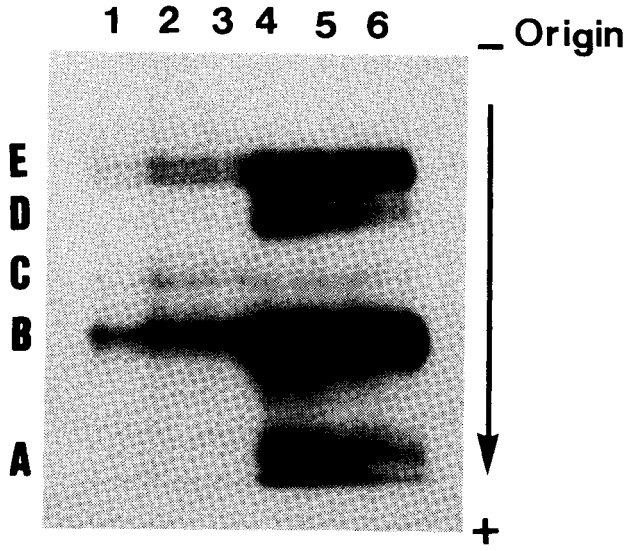

Fig. 1 Zymogram of arginine aminopeptidase in submaxillary glands of individual HR-A ($B_{10}A$) male mice.
Channels 1, 2 and 3=mice fed a Rokumi-gan free diet. Channels 4, 5 and 6=mice fed a 10% Rokumi-gan containing diet. A–E=Arginine aminopeptidase isozyme bands.

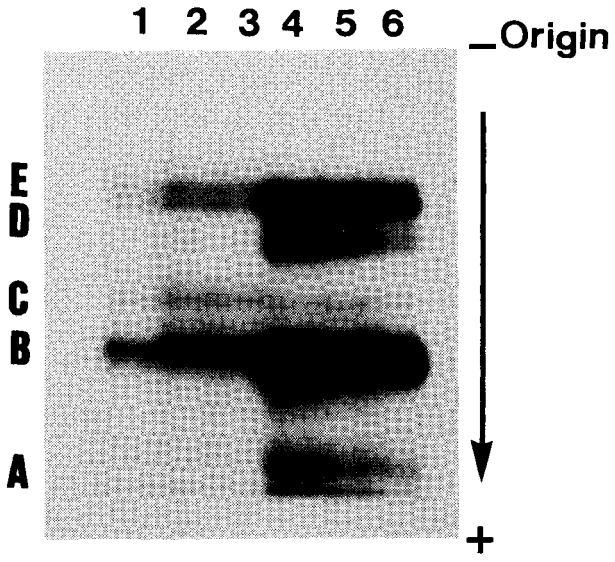

Fig. 2 Zymogram of arginine aminopeptidase in the submaxillary glands of individual HR-B (B_{10}) male mice.
Channels 1, 2 and 3=mice fed Rokumi-gan free diet. Channels 4, 5 and 6=mice fed a Rokumi-gan cotaining diet. A–E=Arginine aminopeptidase isozyme bands.

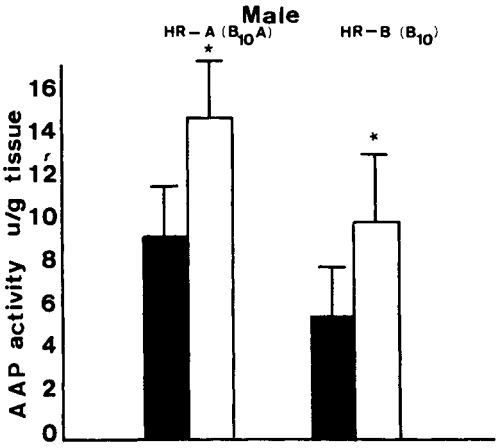

Fig. 3 Arginine aminopeptidase activity in the submaxillary gland of 〔HR-A ($B_{10}A$) and HR-B (B_{10})〕 male mice.
■ Mice fed a Rokumi-gan free diet. □ Mice fed a 10 % Rokumi-gan containing diet. Bars represent means of three experiments, 4—5 mice each. Vertical line indicate SE. $*p<0.05$

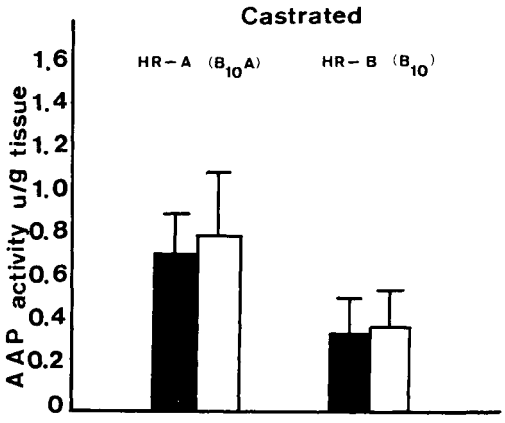

Fig. 4 Arginine aminopeptidase activity in submaxillary gland of 〔HR-4 ($B_{10}A$) and HR-B (B_{10})〕 castrated male mice.
■ Mice fed a Rokumi-gan free diet. □ Mice fed a 10 % Rokumi-gan containing diet. Bars represent means of three experiments, 4—5 mice each. Vertical lines indicate SE.

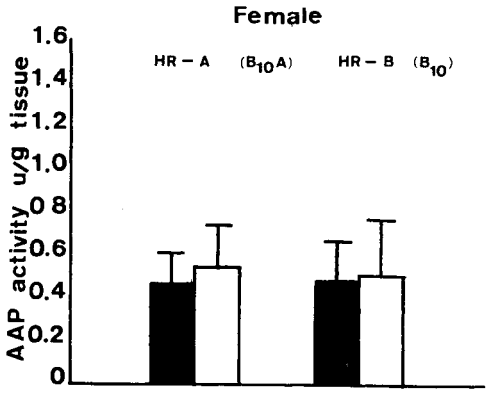

Fig. 5 Arginine aminopeptidase activity in submaxillry gland of 〔HR-A ($B_{10}A$) and HR-B (B_{10})〕 female mice.
■ Mice fed a Rokumi-gan free diet. □ Mice fed a 10 % Rokumi-gan containing diet. Bars represent means of three experiments, 4—5 mice each. Vertical lines indicate SE.

terens of responses of arginine aminopeptidase to Hatimi-ziô-gan and Rokumi-gan in both HR-A and HR-B substrain mice is summarized in Table I. Since Hatimi-ziô-gan treatment resulted in a relative increase in arginine aminopeptidase activity in castrated male mice of both HR-A and HR-B substrains, either the two herbs of Hatimi-ziô-gan, Cinnamomi Cortex

Table I The effects of Hatimi-ziô-gan and Rokumi-gan on arginine aminopeptidase activity in the submaxillary glands of two substrains (HR-A and HR-A) of male, castrated male and female mice.

Oriental Medicines	sex / strain	♂	♂̸	♀
Hatimi-ziô-gan	HR-A ($B_{10}A$)	↑	↑	↑
	HR-B (B_{10})	↑	→	→
Rokumi-gan	HR-A ($B_{10}A$)	↑	→	→
	HR-B (B_{10})	↑	→	→

↑, increase ; →, no effect ; →, decrease. ♂̸ castrated male mice.

and Aconiti Tuber, which are not present in Rokumi-gan, or the combination of additional herbs in Hatimi-ziô-gan act directly on the submaxillary gland to stimulate activity of arginine aminopeptidase.

In the next experiment, we plan to examine the hypothesis that the two herbs Cinnamomi Cortex and Aconiti Tuber found in Hatimi-ziô-gan but not in Rokumi-gan, are responsible for the direct stimulation of arginine aminopeptidase activity.

Acknowledgement

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和文抄録

八味地黄丸に対して応答性を示す2つのマウス集団HR-A系マウス，並びにHR-B系マウスの顎下腺アルギニンアミノペプチダーゼ活性について検討した。

六味丸を10％含有する餌で2週間飼育したところ，両系とも，雄の顎下腺アルギニンアミノペプチダーゼの活性が上昇した。しかし，雌並びに去勢した雄ではその活性の上昇は認められなかった。

このことから，六味丸投与により精巣を介して，アルギニンアミノペプチダーゼの活性上昇がもたらされる可能性が示唆された。

Refrences

1) Kim, Y.K. and Ogita, Z.-I. : Electrophoretic analysis for the androgenic actions of musk. *Japan J. Clin. Chem.* **10** (3), 218-225, 1981
2) Kim, Y.K. and Ogita, Z.-I. : Sexual dimorphism of SDS peptide patterns from the submaxillary glands of mice. *J. Exp. Zool.* **218**, 447-453, 1981
3) Taie, H. and Ogita, Z-I. : A significant effect of Hatimi-ziô-gan on arginine aminopeptidase in mice submaxillary gland. *J. Med. Pharm. Soc. WAKAN-YAKU* **2** (2), 411-417, 1985
4) Taie, H. and Ogita, Z.-I. : Cholorimetric determination of arginine aminopeptidase activity in the submaxillary gland of mice. *Japan J. Clin. Chem.* **14** (6), 405-410, 1985
5) Ogita, Z.-I. and Markert, C.L. : A miniatuarized system for electrophoresis on polyacrylamide gels. *J. Exp. Zool.* **99**, 233-241, 1979
6) Isobe, M. and Ogita, Z.-I. : Electrophoretic analysis of pancreatic proteases and zymogen-activating factors in the mice. *J. Exp. Zool.* **230**, 347-354, 1984
7) Angeletti, R.A., Angeletti, P.U. and Calissano, P. : Testosterone induction of estroproteolytic activity in mouse submaxillary gland. *Biochim. Biophys. Acta* **139**, 372-382, 1967
8) Barka, T. : Biologically active polypeptides in submandibular glands. *J. Histochem. Cytochem.* **28**, 836-859, 1980