A significant effect of Hatimi-ziô-gan on arginine aminopeptidase in mice submaxillary gland

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Abstract

The pharmacological effects of Hatimi-ziô-gan on mice submaxillary glands were examined using arginine aminopeptidase as a marker enzyme. Arginine aminopeptidase, which shows sexual dimorphism in glandular extracts, was separated into 5 bands by vertical polyacrylamide gel electrophoresis. The activity of arginine aminopeptidase in mice submaxillary gland in intact male, female, and castrated male mice of two selected populations fed Hatimi-ziô-gan was monitored. Arginie aminopeptidase activity was increased by feeding a 10% Hatimi-ziô-gan containing diet for two weeks. Two independent mouse populations whose arginine aminopeptidase activity was increased were observed. One was derived from $B_{10}A$ strain, which was named HR-A (Hatimi-ziô-gan responder A) and the increase of enzyme activity was observed in intact male, female, and castrated male mice. The other was derived from B_{10} strain, which was named HR-B (Hatimi-ziô-gan responder B) and the increase of enzyme activity was observed only in intact male mice.

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

Abbreviations BAPNA ; αN-benzoyl-D, L-arginine-*p*-nitroanilide, Hatimi-ziô-gan (Ba-Wai-Di-Huang-Wan) ; 八味地黄丸

Introduction

The submaxillary glands of male and female mice (*Mus musculus*) show both morphological and biochemical sexual dimorphism. Male mice have glands with more extensive convoluted tubles in which the secretory cells are filled with abundant secretory granules.[1-3] The biochemical dimorphism has been revealed by enzymatic and histochemical detection methods.[4]

Arginine aminopeptidase (EC.3.4.11.6.) does show quantitative sexual dimorphism. The amounts of this enzyme, however, vary considerably in different locations within the submaxillary gland. The submaxillary gland, unlike the other salivary glands, expresses uniqe distinctions between males and females. The electrophoretic analysis of sexual dimorphism and the patterns of arginine aminopeptidase isozyme of male and female mice have been previously reported by Kim and Ogita.[5]

Hatimi-ziô-gan (Ba-Wei-Di-Huang-Wan) is one of the Oriental medicines used in management of infertility, enlargement of prostatic glands, ageing, cataract, and diabetes mellitus.[6-9] The effects of Hatimi-ziô-gan on the induction of coupling sites of testosterone receptors in rat

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prostatic glands and on pregnancy have also been reported.[6] Therefore, to clarify the pharmacological effects of Hatimi-ziô-gan, we studied its effects on mouse submaxillary gland by using arginine aminopeptidase as a marker enzyme. The effect of Hatimi-ziô-gan on arginine aminopeptidase activity in submaxillary glands was examined by comparison of the differences in the enzyme activity and its isozyme pattern in males and females, and in males both before and after castration. From the results, we could find out two independent mouse populations having different types of responsiveness to Hatimi-ziô-gan.

Materials and method

Chemicals : αN - Benzoyl - arginine - *p* - nitroanilide HCl (BAPNA) was purchased from Nakarai Chemicals, Ltd. (Tokyo, Japan) ; cellulose acetate membrane (60×220 mm) was purchased from Macherey Nagel Co. (Germany) ; N-1 - naphthylethylenediamine dihydrochloride, acrylamide, N,N-methylene bisacrylamide, and other reagents used were analytical grade products obtained from Wako Pure Chemical Industries (Tokyo, Japan). Hatimi - ziô - gan was obtained from Ohminedo Co., Ltd. (Nara, Japan). Hatimi-ziô-gan consists of eight components ; Rehmanniae Radix 8.0 g, Corni Fructus 4.0 g, Dioscoreae Rhizoma 4.0 g, Alismatis Rhizoma 3.0 g, Hoelen 3.0 g, Moutan Cortex 3.0 g, Cinnamomi Cortex 1.0 g, Aconiti Tuber 1.0 g, in 270 g of diet.

Animals : 8-Week old mice (20-30 g) of ddY, $B_{10}A$, and B_{10} strains of either sex were used. 4-Week old male mice were castrated under anesthesia with nembutal (0.02 mg/body weight ; i.p.). Four weeks after castration, the animals were used. The animals were devided into two groups. The first group was fed a diet containing 10 % Hatimi-ziô-gan for two or eight weeks, while the second group was fed a commen diet (Hatimi-ziô-gan free) as control (Clea Japan, Inc.). 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.

Tissue preparation : Mice were sacrified by cervical dislocation. The submaxillary glands were removed from the mice, washed in ice-cooled 0.9 % NaCl solution to remove traces of blood. These glands were homogenized in 9 volumes of deionized water at 4℃ using a glass homogenizer. The tissue extracts were centrifuged at 20,000×g for 30 minutes at 4℃, and the supernatants were pooled and used for enzymic activity assay and electrophoretic analysis.

Enzymic activity assay: To estimate the activity of arginine aminopeptidase, we used the method of Taie and Ogita,[10] by using αN-benzoyl-D,L-arginine-*p*-nitroanilide as a substrate. A unit of enzymatic activity is defined as the amount of enzyme required to cause a net change of 0.001 absorbance unit after incubation for one minute at 37°C. 43.5 mg of BAPNA was dissolved in 2 ml of dimethylsulfoxide and then diluted with 50 mM phosphate buffer (pH 7.6) to obtain a 1 mM substrate concentration. Incubation at 37℃ was started by the addition of 2 ml of substrate-buffer solution to 1 ml of the tissue extract. The reaction was stopped after 60 minutes by the addition of 1 ml of 20 % perchloric acid. The reaction mixture was centrifuged at 1,500×g for 10 minutes at room temperature. One ml of the resulting supernatant was added to 1 ml of pre-cooled 0.2 % sodium nitrite solution and was kept for 10 minutes in an ice bath. To destroy the excess sodium nitrite, 1 ml of 0.5 % ammonium sulfamate solution was added. After 5 minutes, 2 ml of 0.05 % N - 1 - naphthylethylenediamine dihydrochloride methanol solution was added, and incubated at 37℃ for 30 minutes. The colour was read in a Hitachi model 200/20 spectrophotometer at 546 nm.

Electrophoretic analysis of arginine aminopeptidase : Extracts were mixed with an equal volume of sample diluting solution composed of 5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 8 ml of glycerin, 4 ml of 0.01 % bromophenol blue ethanol solution, and 3 ml of deionized water. Arginine aminopeptidase isozyme was analyzed by the use of a miniaturized electrophoretic apparatus.[11] Electrophoresis was carried out by using 7 % polyacrylamide gel for 2 hours at a constant

current of 1 mA/cm of gel width. The running gel buffer was 0.75 M Tris-HCl buffer (pH 8.3) ; the stacking gel buffer was 0.125 M Tris-HCl buffer (pH 6.8) ; the electrode buffer was 0.125 M Tris-glycine buffer (pH 8.3).

Staining method of arginine aminopeptidase activity : Staining of arginine aminopeptidase activity was accomplished by the method of Isobe and Ogita.[12] Before use, 10 mg of BAPNA was dissolved in 0.5 ml of dimethylsulfoxide by means of shaking and heating in a water bath at 80°C. After the substrate was completely dissolved, 0.1 M phosphate buffer (pH 7.6), was added to make a final volume of 2.5 ml. A cellulose acetate membrane (6×10 cm) was soaked in the substrate solution. The cellulose acetate membrane was placed on the gel and incubated at 37℃ until yellow bands of the *p*-nitroaniline appeared (30-120 minutes). The membrane was then stripped from the gel and soaked for 15 minutes in 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. The membrane was briefly rinsed in a mixture of 40 ml of deionized water containing 500 mg ammonium sulfamate, and then it was soaked in 2N HCl. Arginine aminopeptidase activities were revealed as clear red-purple bands on the cellulose acetate membrane.

Results and discussion

Sexual dimorphism of arginine aminopeptidase in submaxillary glands of mice

Arginine aminopeptidase showed sexual dimorphism in submaxillary glands of mice. In male submaxillary glands of mice, at least five isozyme bands of arginine aminopeptidase were revealed by vertical polyacrylamide gel electrophoresis. The five bands were named A to E, in the order of their mobility from the anode as shown in zymograms (Fig. 1). Arginine amino-

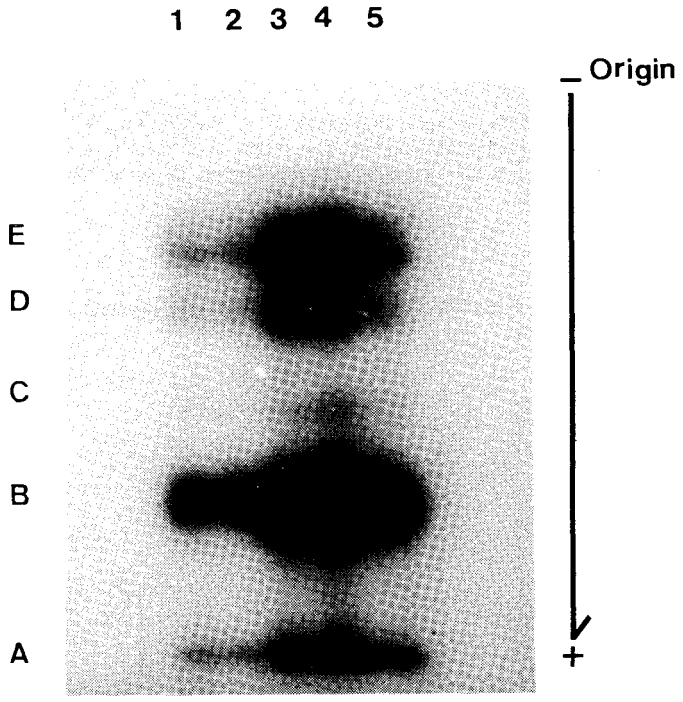

Fig. 1 Zymogram of arginine aminopeptidase in submaxillary glands of individual HR-A ($B_{10}A$) male mice. Channels 1 and 2=HR-A ($B_{10}A$) fed a Hatimi-ziô-gan free diet. Channels 3, 4 and 5=HR-A ($B_{10}A$) fed a 10% Hatimi-ziô-gan containing diet ; A-E=Ariginine aminopeptidase isozyme bands.

peptidase activity in the male was greater than that in the female, which was unable to be visualized in this condition. The male pattern could be converted into the female pattern by castration. These phenomena were also confirmed by enzymic assay using the same substrate

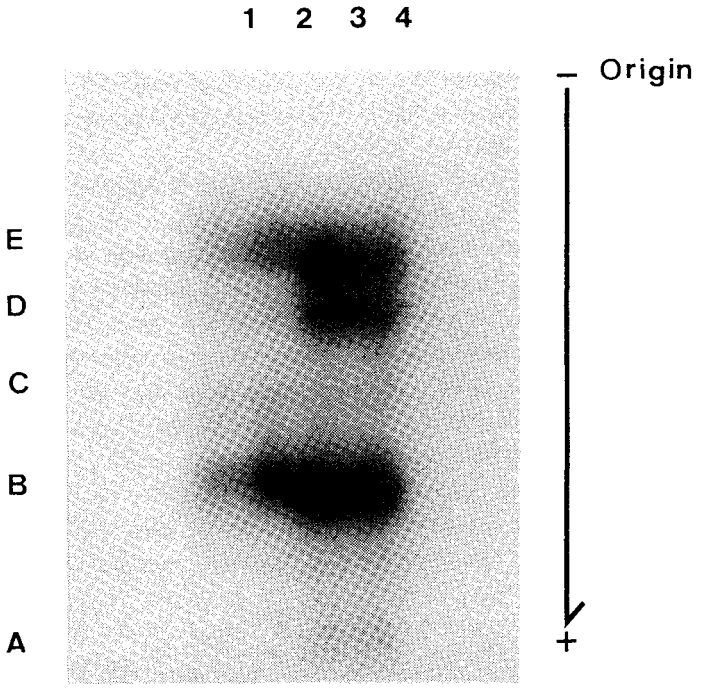

Fig. 2 Zymogram of arginine aminopeptidase in submaxillary glands of individual HR-B (B_{10}) male mice. Channels 1 and 2=HR-B(B_{10}) fed a Hatimi-ziô-gan free diet. Channels 3 and 4=HR-B(B_{10}) fed a 10 % Hatimi-ziô-gan containing diet. A-E=Ariginine aminopeptidase isozyme bands.

Fig. 3 The effect of Hatimi-ziô-gan on arginine aminopeptidase activity in submaxillary gland of [HR-A ($B_{10}A$) and HR-B (B_{10})] male mice.
□ Mice fed a 10 % Hatimi-ziô-gan containing diet. ■ Mice fed a Hatimi-ziô-gan free diet. Bars represent means of four experiments, 4-5 mice each. Vartical lines indicate SE.

αN-benzoyl-D,L-arginine-*p*-nitroanilide.

The effects of Hatimi-ziô-gan on arginine aminopeptidase activity.

Male mice of $B_{10}A$ and B_{10} strains were fed Hatimi-ziô-gan containing diet for two weeks. Arginine aminopeptidase activity in submaxillary gland of these mice was analyzed by electrophoresis. Zymograms of arginine aminopeptidase of submaxillary gland (Figures 1 and 2) showed increase in activity in all active isozyme bands. Spectrophotometric analysis of arginine aminopeptidase in submaxillary gland of male $B_{10}A$ and B_{10} mice after two and eight weeks of feeding Hatimi-ziô-gan containing diet (Figure 3) showed an increase in the activity of the enzyme which increases with feeding time. When the arginine

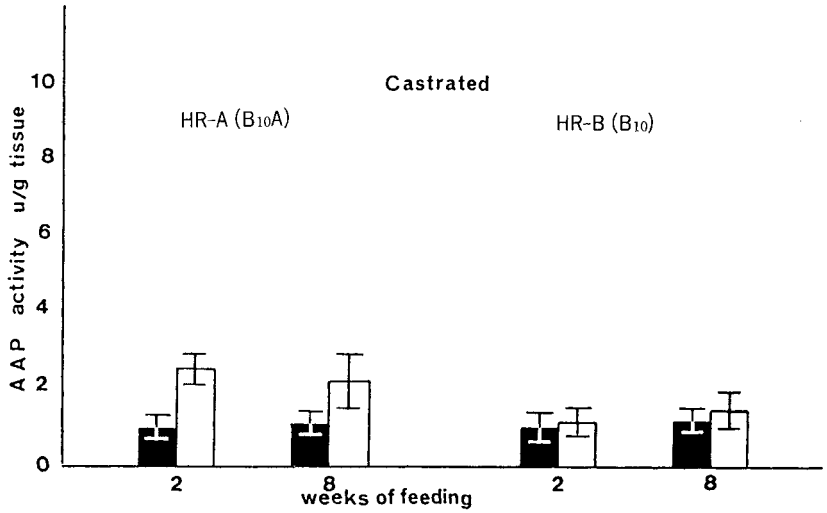

Fig. 4 Arginine aminopeptidase activity in submaxillary gland of [HR-A ($B_{10}A$) and HR-B (B_{10})] female mice feeding a 10 % Hatimi-ziô-gan containing diet □ for period ranging from 2-8 weeks compared with the corresponding controls fed a Hatimi-ziô-gan free diet ■.
Bars represent maens of four experiments, 4-5 mice each. Vartical lines indicate SE.

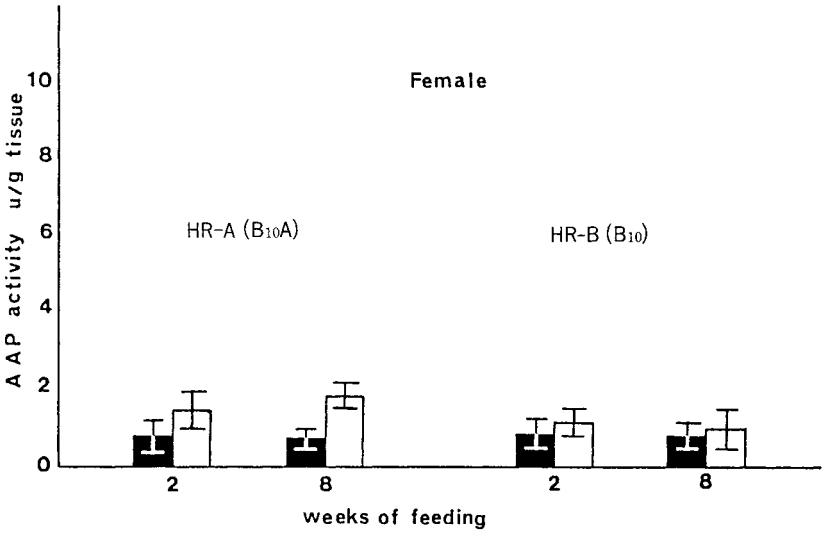

Fig. 5 Arginine aminopeptidase in submaxillary gland of [HR-A ($B_{10}A$) and HR-B (B_{10})] castrated male mice fed a 10 % Hatimi-ziô-gan containing diet □ for period ranging from 2-8 weeks compared with the corresponding controls fed a Hatimi-ziô-gan free diet ■.
Bars represent means of four experiments, 4-5 mice each. Vartical lines indicate SE.

Table I The effect of Hatimi-ziô-gan on arginine aminopeptidase activity in submaxillary gland of different population of mice.

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

aminopeptidase activities of submaxillary glands in female and castrated male mice were analyzed after feeding Hatimi-ziô-gan containing diet for two and eight weeks, the results (Figures 4 and 5) revealed that there is a difference in responsiveness of the two tested strains. These results demonstrated that there were two indepenent populations whose arginine aminopeptidase activity were increased in different mechanism (Table I). One was derived from $B_{10}A$ strain, and the increase of arginine aminopeptidase activity was observed in intact male, female and castrated mice. The other was derived from B_{10} strain, and the increase of arginine aminopeptidase was observed only in intact male mouse. Then, the population drived from $B_{10}A$ was named HR-A (Hatimi-ziô-gan responder A) and the population derived from B_{10} was named HR-B (Hatimi-ziô-gan responder B). These results suggest that there are at least two mechanisms for the effect of Hatimi-ziô-gan on arginine aminopeptidase activity in mice submaxillary glands. That is, one is an indirect mechanism of the effect of Hatimi-ziô-gan through testis, which was observed in HR-B mice. And the other mechanism is a direct effect on submaxillary glands, which was observed in HR-A mice. Consequently the arginine aminopeptidase activity of submaxillary glands in mice may be regulated by at least two kinds of gene.

Two independent mouse populations which responded to Hatimi-ziô-gan in different mechanism, HR-A and HR-B, are derived from congenic strain, $B_{10}A$ and B_{10}, respectively. The genetic difference between HR-A and HR-B is only at the H-2 locus region.[13] It is suggestable that the regulator gene which controls arginine aminopeptidase activity of submaxillary glands in mice is located near the H-2 locus.

We would like to disclose the components of Hatimi-ziô-gan that affect the increase of arginine aminopeptidase activity of mice submaxillary glands, and to clarify the mechanism of increase in arginine aminopeptidase activity by Hatimi-ziô-gan, using HR-A and HR-B mice.

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