Isolation of Bombesin-Like Substances from the Skin of the Frog, *Bombina orientalis*: Its Molecular Heterogeneity and Biological Activity

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= **Abstract** =

Molecular property as well as biological activities of bombesin-like substance (BBS-LS) isolated from the skin of B. *orientalis* in Korea was compared with those of synthetic BBS-14. BBS-LS in the crude extract was applied on a Sephadex G-50, superfine column (1.6×112 cm). On chromatogram, the first peak (3.5% of BBS-LS) was found ahead of synthetic GRP-27, and the second peak (rest) between synthetic GRP-27 and synthetic BBS-14. The main form of BBS-LS was successfully purified by using a column of alkaline alumina followed by sequential gel-filtrations on a column of Sephadex G-10, fine and a column of Sephadex G-50, superfine. Chromatographic analysis of the purified BBS-LS using a column of Sephadex G-50, superfine and reversed phase HPLC revealed that the main form of BBS-LS in the skin of *B. orientalis* could be distinctly different from either BBS-14 or GRP-27 in molecular size. The purified BBS-LS exerted biological activities quite identical to those of synthetic BBS-14. The results of the present investigation indicate that the skin of *B. orientalis* contains BBS-LS composed of two distinct forms. The main form of BBS-LS purified in the present study is heterogenous to both synthetic BBS-14 and GRP-27 in molecular size but identical to BBS in biological activities.

Key Words: Bombesin, gastrin, pancreatic secretion, frog skin, HPLC

INTRODUCTION

A number of biologically active peptides have been found in the skin of several different species of frogs. These include physalaemin (Anastasi et al, 1964), cearulein (Anastasi et al, 1968), phyllocaerulein (Anastasi et al, 1969), bombesin (Erspamer et al, 1970; Anastasi et al, 1971), ranatensin (Nakajima et al, 1970), phyllomedusin (Anastasi et al, 1970) alytesin (Anastasi et al, 1971; Anastasi et al, 1972) and litorin (Anastasi et al, 1975). Bombesin (BBS) is a tetradecapeptide originally isolated from the skin of European frogs belonging to *Bombina* family (Erspamer et al, 1970; Anastasi et al, 1971). BBS is known to stimulate release of gut peptides (Bertaccini et al, 1974; Erspamer et al, 1974; Miyata et al, 1980; Walsh et al, 1981; Ghatei et al, 1982; Vagne et al, 1987), secretion of gastric acid (Bertaccini et al, 1974) and pancreatic enzyme (Erspamer et al, 1974) as well as gastrointestinal motility (Anastasi et al, 1971; Caprilli et al, 1975; Mayer et al, 1982; Reynolds et al, 1986; Vagne et al, 1987). Although BBS has been originally purified from the skin of the frog as a single form (Erspamer et al, 1970; Anastasi et al, 1971), it has been recently reported that 3 different forms of BBS-like immunoreactivity exist in the brain of the frog, *R. catesbiana* (Walsh et al, 1982). Thus, it seems

to be worth while to re-examine molecular heterogeneity of BBS-like substance (BBS-LS) extracted from the skin of *Bombina* family. It has been well documented that another species of *Bombina, B. orientalis*, inhabits Korea (Kang & Yoon, 1975). However, it is uncertain at the present time if the skin of *B. orientalis* contains BBS. Existence of BBS in the skin of *B. orientalis* has been suggested only by means of immunofluorescence technique (Yoshie et al, 1985). Thus, the present study was undertaken to isolate bombesin-like substance from the skin of *B. orientalis* and to examine its molecular property and biological activities in comparison with those of synthetic BBS-14.

METHODS

Isolation of BBS-LS

Purification of BBS-LS : Fresh skins of 300 frogs of *B. orientalis* collected in July and August of 1987 were treated with 5 parts (v/w) of 100% methanol for 3 days at 4℃. Yellow extract was filtered and distilled with a evaporator. Viscous residue remained after distillation was treated with petroleum ether to remove fat and then dissolved in 95% ethanol. Small amount of crude extract dissolved in 95% ethanol was aliquoted for chromatographic analysis. The crude extract in 95% ethanol was applied on a column of alkaline alumina (3×55 cm) which was equilibrated with 95% ethanol and eluted with ethanol-water mixture of decreasing ethanol concentration (90, 85, 80, 75 and 70%, respectively). The concentration of BBS-LS in fractions was determined by radioimmunoassay of BBS. Since most BBS-LS eluted in the 85% ethanol solution, the eluent was lyophilized to apply on a column of Sephadex G-10, fine (1×95 cm) which was previously equilibrated and eluted with 10 mM acetic acid. Absorbance at 280 nm and BBS-LS concentration of each fraction were measured. Fractions containing enough amount of BBS-LS were lyophilized and then applied on a column of Sephadex G-50, superfine (1.6×112 cm) which was also previously equilibrated and eluted with 10 mM acetic acid. Fractions from the column of sephadex G-50, superfine containing enough amount of BBS-LS were again applied on the same column for further purification. BBS-LS purified from the above procedures was lyophilized and then used for determination of molecular heterogeneity and biological activities.

Chromatographic analysis of BBS-LS: Small amount of the crude extract of BBS-LS (equivalent to 250 ng of BBS by radioimmunoassay) as well as the purified BBS-LS (100 ng) was dissolved in 1 ml of 8 M urea solution to apply on a column of Sephadex G-50, superfine (1.6×112 cm) which was previously equilibrated and eluted with 100 mM NH_4HCO_3 containing 0.1% BSA and 0.02% NaN_3. The column was previously calibrated with blue dextran, synthetic porcine gastrin releasing peptide (GRP-27), synthetic BBS-14, and I^{125}. For the purpose of conforming molecular heterogeneity, the purified BBS-LS was subjected to reversed-phase high-performance liquid chromatography (HPLC: model #510, Waters, U.S.A.) using a micro Bondapak C18 column (Waters, U.S.A.) in a size of 3.9×300 mm. The column was equilibrated and eluted with solution of 10 mM CH_3COONH_4 and CH_3CN (7 : 3 v/v) at a constant flow rate of 1 ml/min. Retention times of BBS-LS and synthetic BBS-14 were monitored by spectrophotometry at a 254 nm wave length and radioimmunoassay of BBS.

Radioimmunoassay of BBS

[Tyr^4]-BBS (Sigma, U.S.A.) was labeled using lactoperoxidase (Calbiochem, U.S.A) and NaI^{125} (Amersham, England) according to procedure of Chang et al (1979). [I^{125}-Tyr^4]-BBS was purified with a 10 ml bed column of Sephadex G-15/G-50 fine (8: 2 w/w) followed by a 10 ml bed column of Sp-sephadex, C-25 (Fig. 1). The specific radioactivity of [I^{125}-Tyr^4]-BBS used in the present radio-

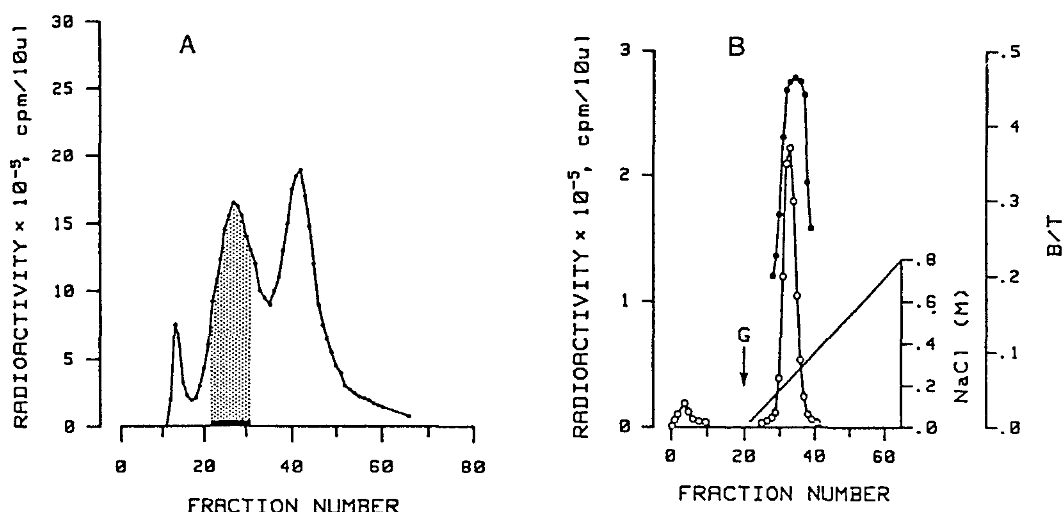

Fig. 1. Purification of labeled [Tyr⁴]-BBS. Gel-filtration was performed on a 10 ml bed column of Sephadex G-15/G-50 fine (8:2 w/w) eluted with 20 mM NaH_2PO_4 (pH 5.5) containing 80 mM NaCl, 0.5 % BSA and 0.02 % NaN_3 (A). Fractions in the 2nd peak (dotted) were pooled and then applied on a 10 ml bed column of Sp-sephadex, C-25 eluted with a linear gradient from 20 to 800 mM NaCl in 20 mM NaH_2PO_4 (pH 5.5) containing 0.5 % BSA and 0.02 % NaN_3 (B). The open and filled circles in "B" represent radioactivity and bound count/total count, respectively.

immunoassay was in the range of 2,220 to 3,450 μCi/nmole as determined by the self-displacement method (Stadil & Rehfeld, 1972). Antiserum raised to porcine GRP-27 in a rabbit was generously given by Dr. W. Y. Chey (The Isaac Gordon Center for Digestive Diseases and Nutrition, The Genesee Hospital, The University of Rochester, Rochester, New York, U.S.A.). The antibody showed full cross-reactivity with BBS-14 but not with other gut peptides. The antibody was used at a final dilution of 1: 167,000 for radioimmunoassay. Mixture of sample, [I^{125}-Tyr^4]-BBS and antibody was incubated for 72 h at 4℃. At the end of the incubation, antibody bound and free counts were separated by centrifugation after adding plasma- and dextran-coated charcoal, and both were counted (Chang et al, 1979). Detection limit of the present radioimmunoassay calculated from the standard curve was 1.85 pM.

Determination of biological activities of BBS-LS

Release of gastrin: Effects of BBS-LS and synthetic BBS-14 on release of gastrin were observed in 17 rats fasted for 24 h. Under light ether anesthesia, 1 ml of blood was collected through the jugular vein 15 min prior to administration of BBS-LS or synthetic BBS-14, and then 1 ml of physiological saline was immediately infused. Fifteen minutes after the administration of BBS-LS or synthetic BBS-14 in a single dose of 0.5 μg/kg through a jugular vein, 2 ml of blood was again sampled through the abdominal aorta. Plasma was separated and stored at −30℃ for furture radioimmunoassay of gastrin (Tai & Chey, 1976)

Pancreatic exocrine secretion: Effects of BBS-LS and synthetic BBS-14 on pancreatic exocrine secretion were observed in 18 rats anesthetized with urethane (1 g/kg) after 24 h fasting. The gas-

troduodenal junction was tightly ligated to prevent passage of gastric juice into the duodenum. The 15 min samples of spontaneous pancreatic secretion were sequentially collected through a tubing inserted into the pancreatic duct while bile juice was diverted into the jejunum. BBS-LS or synthetic BBS-14 in a single dose of 0.1 μg/kg was administered through a jugular vein. The protein concentration in the sample was determined at a 280 nm wave length by spectrophotometry (model# DU-8B, Beckman, U.S.A.) after dilution 1:100 or 1:200 in 0.04 M Tris buffer (pH 7.8). Bovine serum albumin was used as the standard.

Contractility of the ileum, uterus and gall bladder: Effects of BBS-LS and synthetic BBS-14 on contractility of the rat ileum and uterus as well as the gall bladder of the guinea pig were observed. The uterus in diestrus state verified by vaginal smear was used for the present investigation. Spontaneous contractions of the ileal and uterine segment (1 cm in length) as well as the whole gall bladder were recorded in the chamber containing Tyrode solution being aerated with gas mixture of 95 % O_2 and 5% CO_2 at 37℃. BBS-LS or synthetic BBS-14 was administered into the chamber at a concentration of 80 ng/ml for the ileum and gall bladder and 16 ng/ml for the uterus. A force-transducer (UTC3, Gould-Statham, U.S.A.) connected to a recorder (model# R612, Sensormedics, U.S.A.) was used for the recording.

RESULTS

Isolation of BBS-LS

By radioimmunoassay of BBS in the crude extract, it was found that the fresh skin of *B. orientalis* contained 45 μg/g of immunoreactive BBS-LS. In chromatography of small amount of BBS-LS in the crude extract on a column of Sephadex G-50, superfine (1.6×112 cm), two peaks of BBS-LS were shown (Fig. 2). Approximately 3.5 % of BBS-LS was eluted in the first peak with the constant Kav of 0.53, while the remainder of BBS-LS eluted in the second peak with Kav of 0.73. In the previous calibration of the column, Kav of synthetic porcine GRP-27 and synthetic BBS-14 were 0.56 and 0.80, respectively. Sixty-five % of BBS-LS in the crude extract emerged in 85 % ethanol solution after loading on a column of alkaline alumina. When BBS-LS in the 85 % ethanol eluent was applied on a column of Sephadex G-10, as shown in Fig. 3A, BBS-LS eluted in a single peak (dotted) with contaminant. Thus, fractions containing enough amount of BBS-LS (heavy lined) were subjected to apply on a column of sephadex G-50, superfine. BBS-LS also eluted in a single peak even though it was still contaminated. For the purpose of eliminating the contaminant, gel-filtration was repeated on the same column. In the second gel-filtration, BBS-LS eluted in a single peak without contaminant (Fig. 3B). Twenty μg of BBS-like immunoreactivity was obtained from 1 g of the frog skin after final purification.

In chromatographic analysis of the purified BBS-

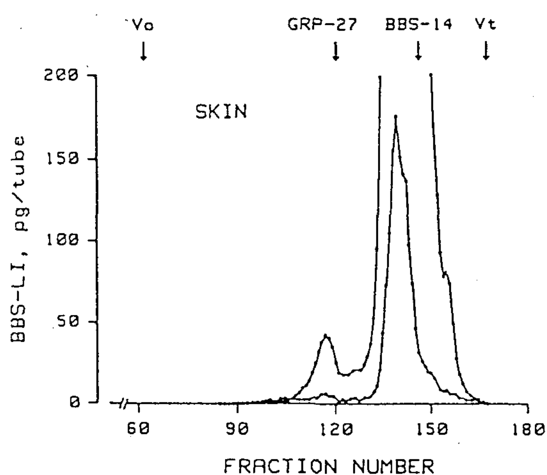

Fig. 2. Chromatograms of BBS-LS in crude extract of the skin of *B. orientalis* on a column of Sephadex G-50, superfine (1.6×112 cm) eluted with 100 mM NH_4HCO_3 containing 0.1 % BSA and 0.02 %NaN_3. The column was previously calibrated with blue dextran, synthetic GRP-27, synthetic BBS-14 and I^{125}. The outer chromatogram was obtained by BBS radiolimmunoassay of fractions with less dilution than the inner one.

LS on the column of Sephadex G-50, superfine (1.6× 112 cm), BBS-LS eluted in a single peak with Kav of 0.73 between synthetic GRP-27 and BBS-14 (Fig. 4B). In reversed phase HPLC, as shown in Fig. 4B, most BBS-LS emerged in a main peak accompanied by several small peaks. When the purified BBS-LS was injected simultaneously with synthetic BBS-14 to the HPLC, BBS-LS was distinctly separated from synthetic BBS-14 with the retention time of 6.78 min. The retention time of synthetic BBS-14 was 5.20 min.

Biological activities of BBS-LS

Release of gastrin: As shown in Fig. 5, at a single dose of 0.5 μg/kg both the purified BBS-LS and

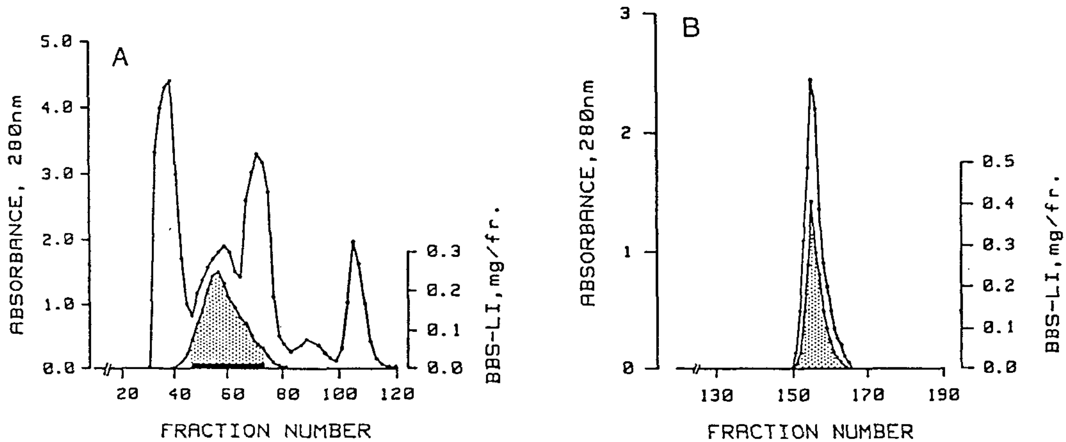

Fig. 3. A: Chromatogram of BBS-LS in 85 % ethanol eluent from alkaline alumina on a column of Sephadex G-10, fine (1×95 cm). Fractions with enough BBS immunoreactivity (heavy lined) were pooled and applied on a column of Sephadex G-50, superfine. B: Chromatogram of BBS-LS at the second time gel-filtration on a column of Sephadex G-50, superfine (1.6×112 cm). Both columns were eluted with 10 mM acetic acid. Absorbance at 280 nm (outer) and BBS immunoreactivity (inner dotted) of fractions were measured.

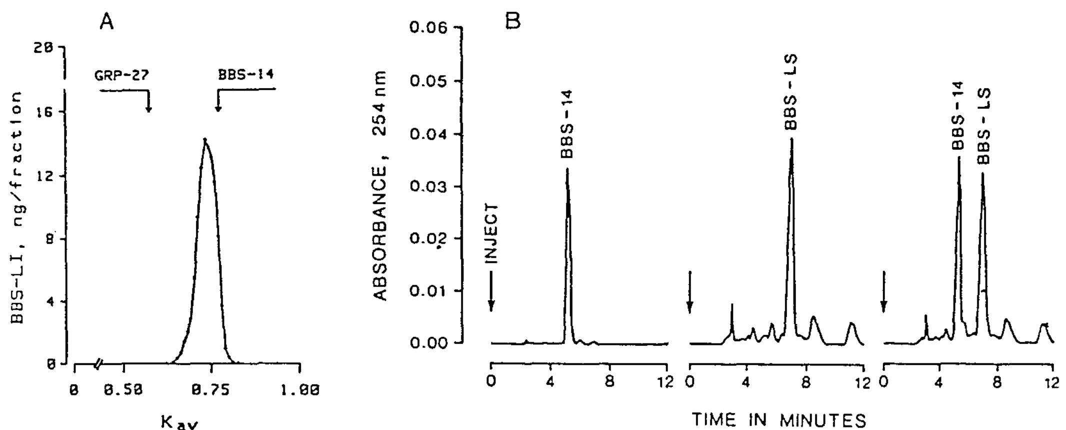

Fig. 4. A: Chromatogram of purified BBS-LS on a column of Sephadex G-50, superfine (1.6×112 cm) eluted with 100 mM NH_4HCO_3 containing 0.1 %BSA and 0.02 %NaN_3. The column was previously calibrated with blue dextran, synthetic GRP-27, systhetic BBS-14 and I^{125}. B: Reversed phase HPLC of purified BBS-LS as well as BBS-14. A micro Bondapack C-18 column was eluted by 10 mM CH_3COONH_4 and CH_3CN (7:3, v/v) at a constant flow rate of 1 ml/min.

synthetic BBS-14 significantly ($p<0.05$, paired t test) elevated the mean plasma concentration of gastrin in fasted anesthetized rats.

Pancreatic exocrine secretion: As shown in Fig. 6, at a single dose of 0.1 μg/kg both the purified BBS-LS and synthetic BBS-14 significantly ($p<0.05$, paired t test) increased flow rate and protein output of spontaneous pancreatic exocrine secretion in fasted anesthetized rats.

Contractility of the ileum, uterus and gall bladder: Both the purified BBS-LS and synthetic BBS-14, as shown in Fig. 7, markedly increased spontaneous contractility of the rat ileum the gall bladder of the guinea pig at a concentration of 80 ng/

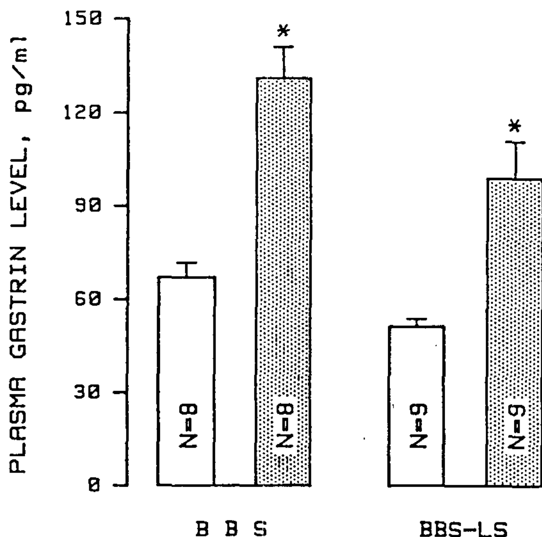

Fig. 5. Effects of purified BBS-LS and synthetic BBS-14 on plasma gastrin concentration in fasted anesthetized rats. Each bar represents mean±S.E. The open bar represents pre-injection value while the dotted bar does post-injection value. The asterisk means the value is significantly different from pre-injection value.

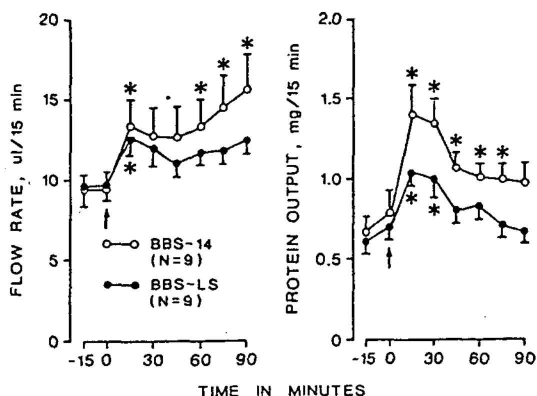

Fig. 6. Effects of purified BBS-LS and synthetic BBS-14 on spontaneous pancreatic exocrine secretion in anesthetized rats. Each point represents mean±S.E. The arrow indicates injection time. The asterisk means the value is significantly different from the value before injection.

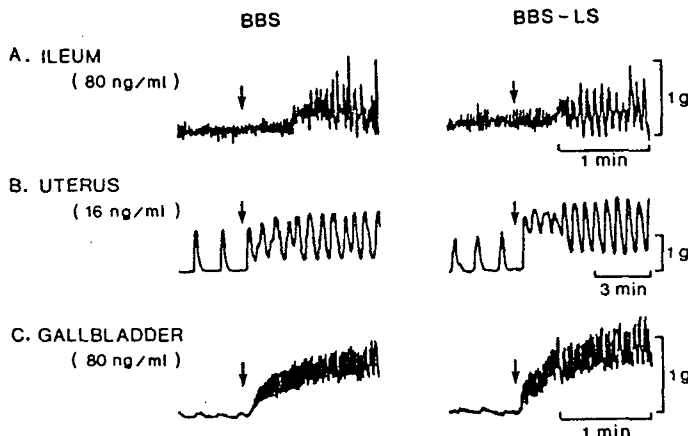

Fig. 7. Effects of purified BBS-LS and synthetic BBS-14 on spontaneous contractility of the rat ileum and uterus ans well as the gall bladder of the guinea pig in vitro. The arrow indicates an event of administration.

ml. The contractility of the rat uterus was also increased by both the purified BBS-LS and synthetic BBS-14 at a concentration of 16 ng/ml.

DISCUSSION

In the present investigation, it is revealed that the skin of *B. orientalis* inhabiting Korea contains considerably large amount of BBS-LS. After treatment of the fresh skin with 100 % methanol, 45 μg/g of BBS-LS was obtained. It has been reported that the fresh skin of *B. bombina* and *variegata variegata* in Europe contains 200~300 μg/g and 400-500 μg/g of BBS activity, respectively (Anastasi et al, 1972). Due to difference in methodology, however, a good comparison of BBS content in the skin among three *Bombina* species is not possible. We used radioimmunoassay, while Anastasi et al (1972) used bioassay on the isolated rat uterus.

The present investigation shows that the skin of *B. orientalis* contains two distinct forms of BBS-LS. Two peaks were observed from chromatographic analysis of the crude extract of BBS-LS on a column of Sephadex G-50, superfine. The first peak, 3.5 % of total BBS-LS, emerged before elution of synthetic GRP-27 while the second peak, 96.5 % of total BBS-LS, emerged in between elutions of synthetic GRP-27 and synthetic BBS-14. The small form appeared to be the main form of BBS-LS isolated from the skin of *B. orientalis* was succesfully purified by the procedures used in the present study. After final purification, 6 mg of BBS-LS was obtained from 100 g of the fresh skin. Unfortunately, however, the purified BBS-LS is revealed to include some contaminants by reversed phase HPLC. Thus, further purification process is required in the future study. The purified BBS-LS appears to possess biological activities very identical to those of synthetic BBS-14. BBS-LS significantly elevated the plasma concentration of gastrin and increased spontaneous exocrine secretion including protein output in fasted anesthetized rats. BBS-LS also stimulated spontaneous contractility of the rat ileum and uterus as well as the gall bladder of the guinea pig in vitro. These results are well known biological activities of synthetic BBS-14 (Anastasi et al, 1971; Bertaccini et al, 1974; Erspamer et al, 1974; Caprilli et al, 1975; Miyata et al, 1980; Walsh et al, 1981; Ghatei et al, 1982; Mayer et al, 1982; Reynolds et al. 1986).

The purified BBS-LS seems to be quite different from synthetic BBS-14 in molecular size. On the chromatogram obtained from the column of Sephadex G-50, superfine, BBS-LS emerged in a different position from that of either synthetic BBS-14 or synthetic GRP-27. Furthermore, it was found in reversed phase HPLC that the purified BBS-LS eluted in a main peak with a retention time distinctly different from that of synthetic BBS-14. When the purified BBS-LS were injected together with synthetic BBS-14 into the micro Bondapack C-18 column in a size of 3.9×300 mm and the column was eluted with 10 mM CH_3COONH_4 and CH_3CN(7:3, v/v) at a constant flow rate of 1 ml/min, BBS-LS and synthetic BBS-14 were well separated with retention times of 6.78 and 5.20 min respectively. Findings of the present study strongly suggest that the skin of *B. orientalis* contains heterogenous forms of BBS-LS that have not been identified yet. Heterogenous forms of BBS-like peptide have been already described in frog tissues and in mammalian tissues. At least 3 different froms of BBS-like immunoreactivity have been demonstrated in the brain and stomach of *Rana catesbiana* (Walsh et al, 1982). In the dog, BBS-like peptides composed of 27, 23 and 10 amino acid residues have been purified from the small intestine and existence of two forms of immunoreactive BBS peptides have been shown in the brain (Reeve et al, 1983). On the other hand, two forms of BBS-like peptides have been also isolated from a metastatic tumor of a bronchial carcinoid in the human (Orloff et al, 1984). Undoubtedly amino acid sequence of the purified BBS-LS should be elucidated in the future study.

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=국문초록=

한국에 서식하는 무당개구리의 피부에서 추출된 Bombesin 유사물질의 분자적 이질성 및 생물학적 활성

한림대학교 의과대학 생리학교실

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Bombesin (BBS)은 유럽에 서식하는 무당개구리(*Bombina bombina*)의 피부로 부터 발견되었으며 이것은 14개의 amino acid로 구성되어 있음이 잘 알려져 있다. 한국에도 유럽의 무당개구리와 종(species)이 다른 무당개구리인 *Bombina orientalis*가 서식함이 알려졌으나 이 무당개구리의 피부에 BBS가 존재하는 지는 확실치 않다. 그러므로 본 실험에서는 한국에 서식하는 무당개구리의 피부로부터 BBS 유사물질(BBS-LS)을 추출하여 분자적 이질성과 생물학적 활성을 측정하고자 하였다. 박리한 무당개구리 피부를 100% methanol로 처리하여 얻은 추출물의 일부를 Sephadex G-50, superfine column에 부하하고 여기에서 얻은 chromatogram을 분석한 결과 BBS-LS의 3.5%는 합성 gastrin releasing peptide (GRP-27) 보다 앞에 용출되었으며 나머지 96.5%는 GRP-27과 합성 BBS-14의 사이에 용출되었다. alkaline alumina column과 Sephadex G-10, fine column 그리고 Sephadex G-50, superfine column을 연속적으로 이용하여 methanol 추출물로 부터 BBS-LS를 정제하였다. 정제된 BBS-LS를 Sephadex G-50, superfine column으로 분석한 결과 합성 GRP-27과 합성 BBS-14 사이에 용출되어 BBS-LS가 이미 알려진 BBS-14와 분자의 크기에 있어서 다른 물질임을 알 수 있었다. 정제된 BBS-LS를 reversed phase HPLC로 분석한 실험은 이러한 결과를 더욱 확실히 뒷받침하였다. 정제된 BBS-LS를 합성 BBS-14와 비교하여 생물학적 활성을 검토한 결과 흰쥐에서 gastrin의 분비, 췌장액의 분비, 회장의 수축을 촉진하고 guinea pig 담낭의 수축을 증가시키는 등 이들 두물질이 매우 유사한 생물학적 활성을 지니고 있음을 발견하였다. 이러한 결과로 보아 한국에 서식하는 무당개구리의 피부는 서로 다른 두가지 형태의 BBS-LS를 함유하고 있으며 그 중에서 96.5%를 차지하는 BBS-LS는 합성 BBS-14와 분자적 이질성을 지니나 유사한 생물학적 활성을 지니고 있음을 알 수 있었다.