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Further Characterization of Bombesin Like Immunoreactivities from the Skin of Korean Fire-bellied Toad, *Bombina orientalis*

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Previously, we have isolated authentic bombesin and another bombesin like peptide named bombesin like immunoreactivity (BLI)-K2 from the skin of Korean fire-bellied toad, *Bombina orientalis.* In the present study, we have newly purified three heterogeneous forms of BLI named BLI-K3, BLI-K4, and BLI-K5 from side fractions obtained in previous isolation of bombesin like peptide. The BLIs were separated into five peaks on a column of C_{18} preparative HPLC. Among them, three minor peaks containing BLI-K3, K4, and K5 were purified by means of sequential chromatography on the columns of SP cation exchange HPLC and C_{18} reverse phase HPLC. The purified BLI-K3 and K4 showed high binding affinity to an anti-bombesin serum (LBE 2G-2) with binding potency of 72 and 95%, respectively, relative to that of bombesin. However, they did not possess any distinctive biological activity of bombesin like peptide. On the contrary, the biological activity of BLI-K5 was similar to that of bombesin but its binding affinity to an anti-bombesin serum was low. The results indicate that three heterogeneous forms of BLI were coexpressed with bombesin and BLI-K2 in the skin of *B. orientalis.* All forms of the purified BLI in the present study were immunologically active but only BLI-K5 possessed the distinctive biological activity of bombesin like peptide.

Key Words: Bombesin, Bombesin like immunoreactivity, Frog skin peptide, Molecular heterogeneity, Gastrin release, Pancreatic exocrine secretion

INTRODUCTION

Bombesin is a tetradecapeptide originally isolated from the skin of European frogs, *Bombina (B) bombina* (Erspamer et al, 1970) and found to have a wide range of biological activities in mammals including stimulation of the release of several gastrointestinal hormones, potent effects on gastrointestinal smooth muscle function and neuromodulation in the central nervous system (Anastasi et al, 1972; Ghatei et al, 1982; Fox & McDonald, 1984; Brown et al, 1977; Rivier & Brown, 1978). At the present time, numerous peptides belonging to the bombesin family have been isolated from amphibian and mammalian tissues (Minamino et al, 1983; Bevin & Zasloff, 1990; Lebacq-Verheyden et al, 1990; Nagalla et al, 1992). Although authentic bombesin is known to exist in only one molecular form in the skin of *B. bombina,* we and others have demonstrated the presence of heterogeneous form of bombesin like peptide in the amphibian tissues (Walsh et al, 1982; Erspamer, 1988; Park et al, 1988; Kwon et al, 1990). Our previous study indicates that bombesin like immunoreactivity (BLI) in the stomach and in the brain of *B. orientalis* emerged with 2 and 3 peaks, respectively on the column of gel permeation chromatography (Park et al, 1988). Furthermore, we have also reported that BLI in the skin of *B. orientalis* were separated into five peaks on a column of C_{18} reverse phase HPLC (Park et al, 1992). Among them, bombesin and another bombesin like peptide named BLI-K2 have

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been purified and their biological activities were examined (Kwon et al, 1990; Park et al, 1992). Because almost of the peptides and amines found in the amphibian skin were shown to have their counterparts in the mammalian tissues (Bevin & Zasloff, 1990), it is quite important to identify and to characterize novel peptides in the amphibian skin. Accordingly, in the present study, we have newly purified three heterogeneous forms of BLI named BLI-K3, BLI-K4, and BLI-K5 from side fractions obtained in previous isolation of bombesin like peptide. The biological and immunological activities of purified BLIs were also examined in comparison to those of authentic bombesin.

METHODS

Materials

Bombesin, lactoperoxidase, bovine serum albumin (Fr. V), bovine trypsin (type III), dinitrosalysilic acid, N-benzoyl-L-tyrosine ethyl ester, Trizma base were purchased from Sigma Chemical Co. (St. Louis, USA). [tyr^4]-Bombesin was purchased from Penninsula Lab. Inc. (Belmont, USA). Sephadex G-10, Sephadex G-25, C_{18} reverse phase HPLC column (ODS-120T), SP ion exchange HPLC column (SP-5PW), gel permeation HPLC column (TSK G2000-SW) were purchased from LKB/Pharmacia (Uppsala, Sweden). NaI^{125} was purchased from Amersham (Buckinghamshire, England). All other chemicals were of the highest analytical or HPLC grade available. 400 frogs of *B. orientalis* were collected at rural area nearby Chunchon city, Kangwon-Do, Korea. Sprague-Dawley rats weighing about 250 g were supplied from the experimental animal center at Hallym University.

Purification of BLI

Bombesin like immunoreactivity (BLI) was extracted from the skins of 400 frogs (wet weight 420 g) with 100% methanol and partially purified by using sequential column chromatographys of alkaline alumina, Sephadex G-10 and C_{18} preparative HPLC according to the method described previously (Park et al, 1992). C_{18} HPLC fractions containing enough amount of BLI-K3, K4 and K5 were lyophilized and then injected into a 7.5×75 mm SP-5PW column equilibrated with 20 mM NaH_2PO_4, pH 5.5, connected to the LKB HPLC system, and eluted with a linear gradient from 0 to 500 mM NaCl in 20 mM NaH_2PO_4, pH 5.5, respectively. The active fractions were combined and removed salts by using a Sephadex G-25 column chromatography, respectively. Each lyophilized BLI was injected into a 4.6×250 mm C_{18} analytical HPLC column equilibrated with 0.1% trifluoroacetic acid, 10% acetonitrile and eluted with a linear gradient from 10 to 50% acetonitrile in 0.1% trifluoroacetic acid. Finally purified BLI-K3, K4, and K5 were lyophilized respectively in small volumes and stored frozen at -70°C.

Radioimmunoassay

Throughout the whole steps of purification, radioimmunoassay (RIA) was performed to monitor the peaks containing BLI in column fractions. Specific bombesin RIA was performed according to the method described previously (Park et al, 1989). Lactoperoxidase-catalyzed iodination of [tyr^4]-bombesin was carried out at room temperature. ^{125}I-[tyr^4]-Bombesin was purified by injection into a C_{18} reverse phase column attached to HPLC system (LKB, Sweden). The specific radioactivity of purified ^{125}I-[tyr^4]-bombesin used in the present study was 2,400 Ci /nmole as determined by the self-displacement method (Stadil & Rehfeld, 1972). The anti-bombesin serum (LBE 2G-2) used in the present study has been shown to have almost absolute specificity for bombesin and negligible cross-reactivity with other bombesin family peptides including gastrin releasing peptide (GRP), ranatensin and neuromedins (Kwon et al, 1994). Plasma gastrin concentration was determined by gastrin RIA according to the method described previously (Tai & Chey, 1976).

Biological activity of BLI

Release of gastrin: Fasted rats were anesthetized with a single intraperitoneal injection of 25% urethane at a dose of 5 ml/kg of body weight. One ml blood was collected through the jugular vein 15 min prior to administration of purified BLI or bombesin, and then one ml physiological saline was immediately infused. Thirty min after the administration of BLI or bombesin through a jugular vein, 2 ml blood was again sampled. Two doses of BLI or bombesin were used for administration in the present study. One was a 0.5 μg/kg on the basis of peptide weight,

and another was a 0.5 μg BLI/kg on the basis of BLI content determined by RIA. i.e, 0.5 μg BLI was equivalent to 0.50 μg bombesin, 0.69 μg BLI-K3, 0.53 μg BLI-K4, and 1.85 μg BLI-K5, respectively (Fig. 5). Plasma was separated and stored at -30°C for gastrin RIA.

Pancreatic exocrine secretion: Surgery for the collection of rat pancreatic juice was performed according to the method described previously (Park et al, 1989). The 30 min sample of spontaneous pancreatic secretion was collected through a tubing inserted into the pancreatic duct before administration of BLI or bombesin. Another 30 min sample of pancreatic juice was sequentially collected after administration of BLI or bombesin. Two doses of BLI or bombesin were also used for administration; 0.1 μg/kg and 0.1 μg BLI/kg. The concentrations or enzyme activities of protein, bicarbonate, amylase, and chymotrypsin in pancreatic juices were determined according to the method described previously (Hummel, 1959; Rick & Stegbauer, 1974; Park et al, 1992).

Immunoreactivity of BLI

The immunoreactivity was assessed by measuring the ability of purified BLI to compete with ^{125}I-[tyr^4]-bombesin for binding sites of an anti-bombesin serum (LBE 2G-2) with a titer of 1:167,000 according to the method described previously (Chang & Chey, 1983). The extent of cross-reactivity with an anti-bombesin serum was expressed as the half-saturation concentration.

Statistical analysis

All data are illustrated as means ± SE. The statistical analysis was evaluated by the Student's *t*-test. The difference was considered significant when $P<0.05$.

RESULTS

Purification of BLI-K3, K4, and K5

After C_{18} preparative HPLC, BLIs were separated into five peaks which were designated BLI-K1 ~ BLI-K5, respectively (Fig. 1). As previously reported, the main form of BLI in the skin of *B. orientalis*, BLI-K1, has confirmed to be an authentic bombesin

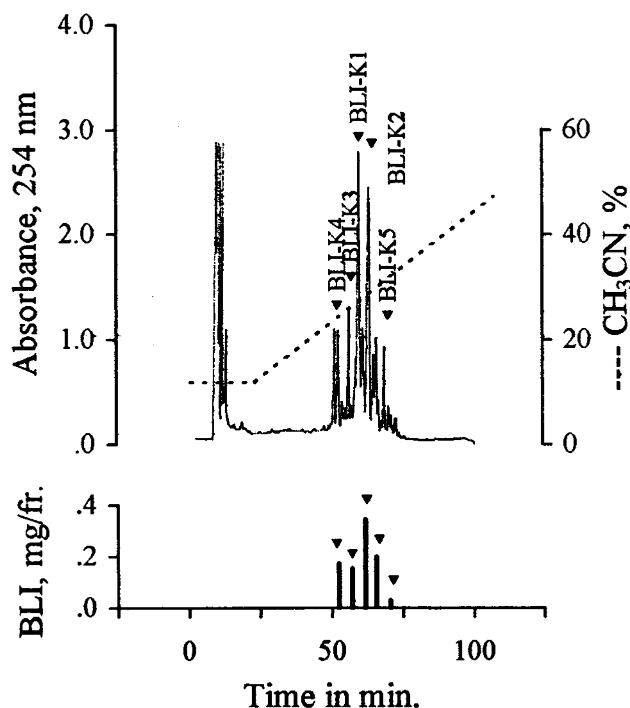

Fig. 1. C_{18} reverse phase HPLC profile of bombesin like immunoreactivity (BLI). Sephadex G-10 fractions with enough BLI were pooled and injected into a C_{18} preparative HPLC column equilibrated with 0.1% trifluoroacetic acid, 10% acetonitrile and eluted with a linear gradient from 10 to 50% acetonitrile in 0.1% trifluoroacetic acid for 100 min. Upper pannel represents the absorbance at 254 nm and bars in lower pannel represent BLI content in each fraction. Each arrow indicates the peak fraction of BLI.

(Park et al, 1992). The BLI-K2 has also been purified and characterized but its amino acid sequence remains unknown (Kwon et al, 1990). The present purification was concerned with BLI-K3, K4, and K5. The BLI-K3 and K4 were less hydrophobic than bombesin on a reverse phase column as shown in Fig. 1. However, the BLI-K5 showed more hydrophobic than bombesin. After lyophilization of BLI containing C_{18} preparative HPLC fractions, the resulting BLI was further separated by a cation exchange HPLC on column of SP-5PW. The BLI-K3, K4, and K5 were eluted on column of SP-5PW at concentrations of 120, 125, and 150 mM NaCl, respectively (data not shown). Final furification of BLI was achieved by a reverse phase HPLC on C_{18} analytical column. After performing C_{18} analytical HPLC, each form of BLI was eluted in a single peak, respectively. The purity of BLI was confirmed by using C_{18} analytical HPLC,

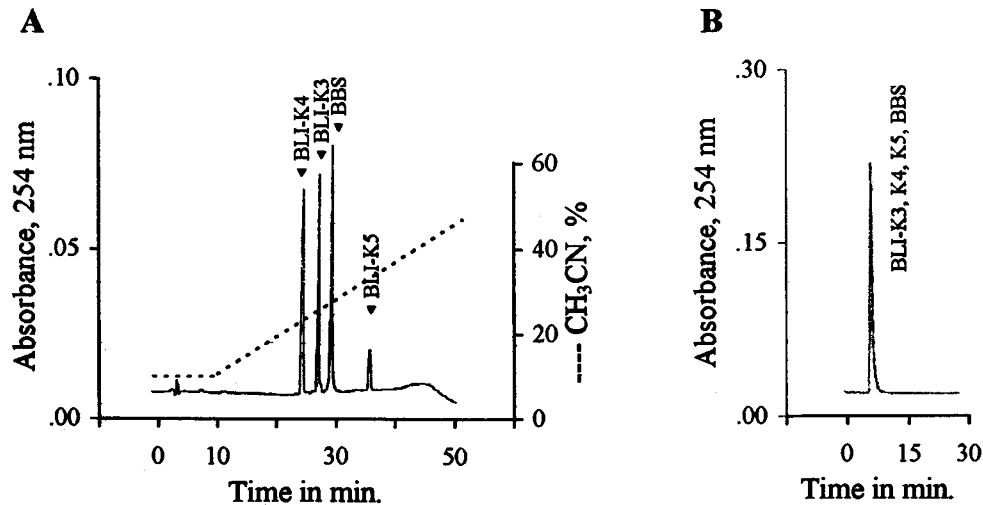

Fig. 2. Comparison of three purified bombesin like immunoreactivities (BLIs) with bombesin (BBS) by using C_{18} analytical HPLC (A) and gel permeation HPLC (B). The mixture of three purified BLIs and BBS were injected into either C_{18} HPLC column (ODS-120T) or gel permeation HPLC column (TSK G2000-SW). Each arrow indicates the peak fraction of BLI.

SP-5PW HPLC, and gel permeation HPLC. To confirm the molecular heterogeneity of purified BLIs, the mixture of three forms of BLIs and bombesin were injected into C_{18} reverse phase HPLC column and gel permeation HPLC column, respectively. As shown in Fig. 2, peptide components in the mixture were well separated from each other on a C_{18} HPLC column but not separated on a gel permeation HPLC column, suggesting that purified BLIs were similar to bombesin in molecular size but different from each other in respect to their molecular hydrophorbicity or polarity. Final amounts of BLI-K3, K4, and K5 purified from the skins of 400 frogs were 154.8, 153.8, and 17.1 μg, respectively.

Biological activity of BLI-K3, K4, and K5

Release of gastrin: As shown in Fig. 3, administration of bombesin at a dose of 0.5 μg/kg significantly elevated the plasma gastrin level from the basal value of 65.67±13.08 pg/ml to 145.89±22.73 pg/ml. In contrast, plasma gastrin concentration was not significantly changed by the administration of either BLI-K3, K4, or K5 at the same dose. However, when rats were given purified BLIs at a dose of 0.5 μg BLI/kg on the basis of BLI content, the BLI-K5 significantly elevated the plasma gastrin concentration from the basal value of 63.57±7.72 pg/ml to 135.58±18.83 pg/ml whereas both BLI-K3 and K4 did not.

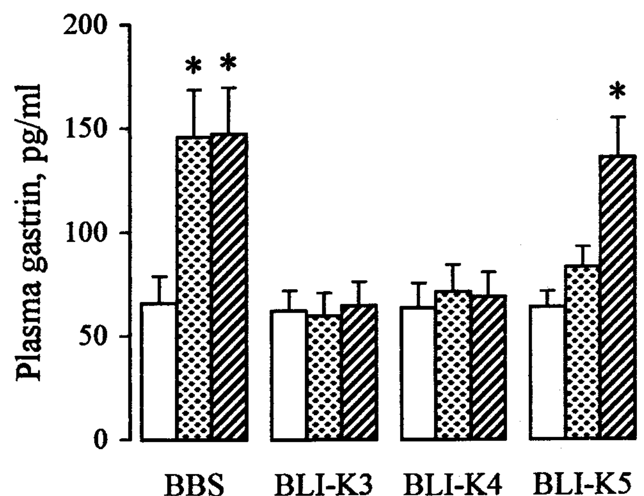

Fig. 3. Effects of three purified bombesin like immunoreactivities (BLIs) and bombesin (BBS) on the mean plasma gastrin concentration in fasted anesthetized rats. The open bars represent the values of basal state. Purified BLIs or BBS were administrated in two doses of 0.5 μg/kg on the basis of peptide weight (dotted bars) and 0.5 μg BLI/kg on the basis of BLI content (hatched bars). Each bar represents means±SE obtained from 7 rats. Asterisks indicate the value is significantly different from that of basal state.

Pancreatic exocrine secretion: As shown in Fig. 4 and Table 1, administration of bombesin at a dose

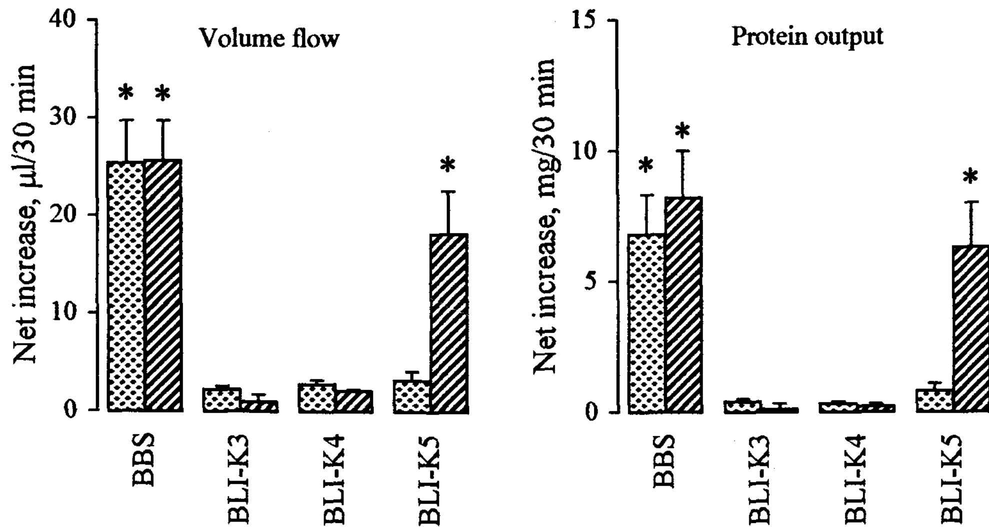

Fig. 4. Effects of three purified bombesin like immunoreactivities (BLIs) and bombesin (BBS) on pancreatic volumn flow and protein output in fasted anesthetized rats. Each bar represents the value of net increase compared with that of basal state for 30 min. The purified BLIs or BBS were administrated in two doses of 0.1 μg/kg on the basis of peptide weight (dotted bars) and 0.1 μg BLI/kg on the basis of BLI content (hatched bars). Each bar represents means±SE obtained from 5~7 rats. Asterisks indicate the value is significantly different from that of basal state.

Table 1. Effects of three purified bombesin like immunoreactivities (BLIs) and bombesin (BBS) on pancreatic bicarbonate output, amylase output, and chymotrypsin output in fasted anesthetized rats

Peptide	(dose)	ΔBicarbonate output (mEq/30 min)	ΔAmylase output (KU/30 min)	ΔChymotrypsin output (mU/30 min)
BBS	(0.1 μg/kg)	1.3±0.3*	2.5±0.5*	30.5±5.6*
	(0.1 μg BLI/kg)	1.1±0.2*	2.3±0.5*	36.8±6.2*
BLI-K3	(0.1 μg/kg)	0.3±0.1	0.1±0.1	−0.4±0.3
	(0.1 μg BLI/kg)	0.1±0.1	0.1±0.1	0.3±0.5
BLI-K4	(0.1 μg/kg)	0.3±0.1	0.1±0.1	0.3±0.3
	(0.1 μg BLI/kg)	0.2±0.1	−0.1±0.1	−0.5±1.2
BLI-K5	(0.1 μg/kg)	0.2±0.1	0.2±0.1	4.2±1.3
	(0.1 μg BLI/kg)	1.3±0.3*	1.8±0.4*	28.4±5.9*

Each value represents the net increase compared with that of basal state for 30 min. The purified BLIs or BBS were administrated in two doses of 0.1 μg/kg on the basis of peptide weight and 0.1 μg BLI/kg on the basis of BLI content. Each value represents means±SE obtained from 5~7 rats. An asterisk indicates the value is significantly different from that of basal state.

of 0.1 μg/kg resulted in a significant increase in pancreatic volume flow, protein output, bicarbonate output, amylase output, and chymotrypsin output. In contrast, pancreatic secretion was not influenced by the administration of either BLI-K3, K4, or K5 at the same dose. However, when rats were given purified BLIs at a dose of 0.1 μg BLI/kg on the basis of BLI content, the BLI-K5 resulted in a significant increase in pancreatic exocrine secretion whereas both BLI-K3 and K4 did not.

Immunoreactivity of BLI-K3, K4, and K5

The immunoreactivity of purified BLIs with an

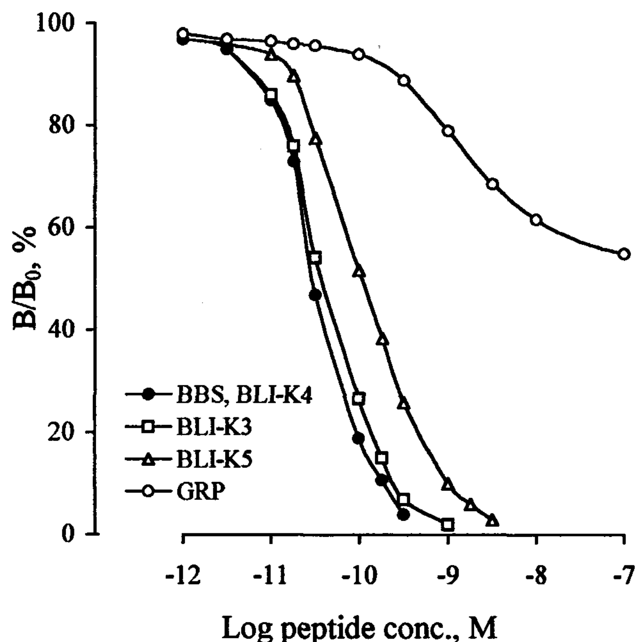

Fig. 5. Cross-reactivity of three purified bombesin like immunoreactivities (BLIs) and bombesin (BBS) with an anti-bombesin serum (LBE 2G-2). Extent of the cross-reactivity was expressed as tracer binding ratio at various peptide concentrations (B) comparing with that of zero concentration (B_0). GRP; gastrin releasing peptide.

anti-bombesin serum (LBE 2G-2) is shown in Fig. 5. The relative immunoteactivies with LBE 2G-2 were bombesin >BLI-K4 >BLI-K3 >BLI-K5. The half-saturation concentrations of bombesin, BLI-K3, K4, and K5 were 40.0, 55.2, 42.2, and 147.9 pM, respectively. Therefore, the binding potency of BLI-K3, K4, and K5 to LBE 2G-2 could be estimated as 72, 95, and 27% of bombesin, respectively.

DISCUSSION

Previously, we have suggested that there are at least five heterogeneous forms of bombesin like immunoreactivity (BLI) in the skin of *B. orientalis* inhabiting Korea (Park et al, 1992). Among them, two forms of BLI, authentic bombesin and BLI-K2 were successfully purified and characterized (Kwon et al, 1990; Park et al, 1992). The existence of authentic bombesin in the skin of *B. orientalis* have been already confirmed by immunofluorescence staining (Yoshie et al, 1985) and by cloning of cDNA encoding amphibian bombesin (Spindel et al, 1990). However, little is known about the existence of heterogeneous forms of BLI in the frog skin.

In the present study, we have purified three new forms of BLI named BLI-K3, K4, and K5 in the skin of *B. orientalis*. Among them, only BLI-K5 possessed distinctive biological activity of bombesin like peptide as well as immunological activity. Heterogeneous forms of BLI have been described in frog tissues and in mammalian tissues. Three different forms of BLI have been demonstrated in the brain and in the stomach of *B. orientalis* and *Rana catesbiana*, respectively (Walsh et al, 1982; Park et al, 1988). Recently, Nagalla et al (1992) have purified frog gastrin releasing peptide (fGRP)-29 and GRP-10 from the gut extracts of *B. orientalis*. A typical bombesin like nonapeptide (pGlu[1]-bombesin$_{6\text{-}14}$) have been identified in the skin of a *Rana* species inhabiting Papua New Guinea (Erspamer, 1988). In mammalian tissue, bombesin like peptides composed of 27, 23, and 10 amino acid residues have been purified and sequenced from the small intestine of dog (Reeve et al, 1983). Our observation that *B. orientalis* skin contains several forms of BLI seems to be consistent with these lines of evidence for molecular heterogeneity of bombesin like peptides.

The purified BLI-K3 and K4 share similar characteristics in terms of biological activity and immunological activity. These peptides were less hydrophobic than bombesin on a column of C_{18} reverse phase HPLC (Fig. 1) and showed high affinity to an anti-bombesin serum (Fig. 5). However, BLI-K3 and K4 did not show any distinctive biological activity of bombesin like peptide. Neither plasma gastrin concentration nor pancreatic exocrine secretion including volume flow, bicarbonate output, and pancreatic enzyme output was affected by administration of BLI-K3 or K4 in anesthetized rats. Studies on the structure-activity relationship of bombesin revealed that C-terminal nonapeptide of bombesin is essential in eliciting the full spectrum of bombesin activity (Erspamer et al, 1988). The amidated C-terminal of bombesin (-Met-NH_2) has also been described as an essential residue for high affinity binding and biological activity of bombesin. The binding potency and biological activity of bombesin were markedly decreased by extension or deamidation of C-terminus (Mervic et al, 1991). Accordingly, the purified BLI-K3 and K4 are assumed as bombesin like peptides in which C-terminal amino acids indispensable for biological activity may be substituted, modified or deamidated.

The amino acid sequencing of purified BLIs is absolutely required to establish the structure-activity relationship of BLI. Because BLI-K3 and K4 showed high affinity to an anti-bombesin serum, it was assumed that structural homology of BLI-K3 and K4 with bombesin still retains high. Therefore, we examined the antagonistic effect of BLI-K3 or K-4 on bombesin-stimulated pancreatic exocrine secretion in anesthetized rats. However, neither BLI-K3 nor K4 affected on bombesin-stimulated pancreatic volume flow and protein output (data not shown).

In contrast to BLI-K3 and K4, the purified BLI-K5 appeared to possess biological activities very identical to those of bombesin. When BLI-K5 was administrated in the same dose as bombesin on the basis of BLI content, the BLI-K5 significantly elevated the plasma gastrin concentration and increased spontaneous pancreatic exocrine secretion including volume flow, bicarbonate output, and enzyme output in fasted anesthetized rats (Fig. 3, 4, Table 1). These results agree well with characteristic biological activities of bombesin (Bertaccini et al, 1974; Konturek et al, 1976; Varga et al, 1988). However, the binding affinity of BLI-K5 to an anti-bombesin serum was low, 27% of bombesin (Fig. 5). The anti-bombesin serum, LBE 2G-2, used in the present study have been shown to have almost absolute specificity for bombesin and negligible cross-reactivity with other bombesin family peptides including GRP, ranatensin and neuromedins. It is therefore indicated that this anti-bombesin serum is specific for N-terminal or whole amino acid sequence of bombesin (Kwon et al, 1994). Accordingly, the purified BLI-K5 is assumed as a bombesin like peptide in which nonessential amino acids for biological activity on N-terminal or middle part of bombesin may be substituted with more hydrophobic amino acids.

In summary, three heterogeneous forms of BLI named BLI-K3, K4, and K5 were newly purified and characterized from the skin of *B. orientalis* in addition to our previous purification of bombesin and BLI-K2. Among the purified BLIs, only BLI-K5 possessed the characteristic biological activities of bombesin family peptide. The amino acid sequence of purified BLIs remains unknown at the present time.

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REFERENCES

Anastasi A, Erspamer V, Bucci M. Isolation and amino acid sequences of alytesin and bombesin, two analogues active tetradecapeptides from the skin of European discoglossid frogs. *Arch Biochem Biophys* 148: 443−446, 1972

Bertaccini G, Erspamer V, Melchiorri P, Sopranzi N. Gastrin release by bombesin in the dog. *Br J Pharmac* 52: 219−225, 1974

Bevins CL, Zasloff M. Peptides from frog skin. *Annu Rev Biochem* 59: 395−414, 1990

Brown MR, Rivier JE, Vale W. Bombesin affects the central nervous system to produce hyperglycemia in rats. *Life Sci* 21: 1729−1734, 1977

Chang TM, Chey WY. Radioimmunoassay of cholecystokinin. *Dig Dis Sci* 28: 456−468, 1983

Erspamer V, Erspamer G, Inselvini M. Some pharmacological actions of alytesin and bombesin. *J Pharm Pharmac* 22: 875−876, 1970

Erspamer V. Discovery, isolation, and characterization of bombesin like peptide. In: Tache Y, Melchiorri P, Negri L ed, *Bombesin-like peptides in health and disease*, The New York Academy of Sciences, New York, p3−9, 1988

Erspamer GF, Severini C, Erspamer V, Melchiorri P, Delle Fave G, Nakajima T. Parallel bioassay of 27 bombesin-like peptides on 9 smooth muscle preparations. Structure-activity relationships and bombesin receptor subtypes. *Regul Peptide* 21: 1−11, 1988

Fox JET, McDonald TJ. Motor effects of gastrin releasing peptide and bombesin in the canine stomach and small intestine. *Life Sci* 35: 1667−1673, 1984

Ghatei MA, Jung RT, Stevenson JC, Hillyard CJ, Adrian TE, Lee YC, Christofides ND, Sarson DL, Mashiter K, MacIntyre I, Bloom SR. Bombesin: Action on gut hormones and calcium in man. *J Clin Endocrin Metabol* 54: 980−985, 1982

Hummel BCW. A modified spectrophotometric determination of chymotrypsin, trypsin and thrombin. *Can J Biochem Physiol* 37: 1393−1402, 1959

Konturek SJ, Krol R, Tasler J. Effect of bombesin and related peptides on the release and action of intestinal hormones on pancreatic secretion. *J Physiol* 257: 663−672, 1976

Kwon HY, Y Kim, Park HJ. Purification and biological activities of bombesin like immunoreactivity from skin of the frog, *Bombina orientalis* in Korea. *Kor J Physiol* 24: 363−375, 1990

Kwon HY, Lee YL, Park HJ. Production and characteri-

zation of specific antibodies to bombesin. *Kor J Physiol* 28: 91−97, 1994

Lebacq-Verheyden AM, Trepel J, Sausville EA, Battey JF. Bombesin and gastrin releasing peptide: Neuropeptide, secretogogues, and growth factors. In: Born GVR, Cuatrecasas P ed, *Handbook of experimental pharmacology*, Springer-Verlag, Berlin, p71−124, 1990

Mervic MM, Moody TW, Komoriya A. A structure function study of C-terminal extensions of bombesin. *Peptides* 12: 1149−1151, 1991

Nagalla SR, Gibson BW, Tang D, Reeve JR, Spindel ER. Gastrin releasing peptide (GRP) is not mammalian bombesin. *J Biol Chem* 267: 6916−6922, 1992

Park HJ, Lee YL, Kwon HY, Shin WI, Suh SW. Molecular heterogeneity of bombesin-like immunoreactivity extracted from tissues of Korean frog, *Bombina orientalis. hallym Non Mun Jib* 6: 121−134, 1988

Park HJ, Lee YL, Kwon HY, Shin WI, Suh SW. Isolation of of bombesin-like substances from skin of the frog, *Bombina orientalis*: Its molecular heterogeneity and biological activity. *Kor J Physiol* 23: 79−87, 1989

Park HJ, Lee YL, Kwon HY, Kim Y, Yu IJ, Marshak DR. Studies on biologically active bombesin-like peptides in frog (*Bombina orientalis*) inhabiting Kangwon-Do. *Kor Biochem J* 25: 269−275, 1992

Reeve JR, Walsh JH, Chew P, Clark B, Hawke D, Shively JE. Amino acid sequences of three bombesin-like peptides from canine intestine extracts. *J Biol Chem* 258: 5582−5588, 1983

Rick W, Stegbauer HP. α-Amylase, measurement of reducing groups. In: Bergmeyer HU ed, *Methods of enzymatic analysis.* 2nd ed. Verlag Chemie, Weiheim, p885−915, 1974

Rivier JE, Brown MR. Bombesin, bombesin analogues, and related peptides: Effects on thermoregulation. *Biochemistry* 17: 1766−1771, 1978

Spindel ER, Gibson BW, Reeve JR, Kelly M. Cloning of cDNAs encoding amphibian bombesin: Evidence for the relationship between bombesin and gastrin-releasing peptide. *Proc Natl Acad Sci* 87: 9813−9817, 1990

Stadil F, Rehfeld JF. Preparaion of ^{125}I-labelled synthetic human gastrin-I for radioimmunoanalysis. *Scand J Clin Invest* 30: 361−368, 1972

Tai HH, Chey WY. Simultaneous radioimmunoassay of secretin and gastrin. *Anal Biochem* 74: 12−24, 1976

Varga G, Papp M, Dobronyi I, Scarpignato C. Effect of bombesin and its mammalian counterpart, GRP, on exocrine pancreas in the rat. *Digestion* 41: 229−236, 1988

Walsh JH, Lechago J, Wong HC, Rosenquist GL. Presence of ranatensin-like and bombesin-like peptides in amphibian brains. *Regul Peptide* 3: 1−13, 1982

Yoshie S, Iwanaga T, Fujita T. Coexistence of bombesin and 5-hydroxytryptamine in the cutaneous gland of the frog, *Bombina orientalis. Cell Tissue Res* 239: 25−29, 1985