

# Immunohistochemistry of Endocrine Cells in the Alimentary Tract of the Tree Frog, *Hyla arborea japonica*

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The regional distribution and relative frequencies of endocrine cells were studied immunohistochemically (PAP methods) in the alimentary tract of the tree frog, *Hyla arborea japonica*, using specific antisera against serotonin, somatostatin, bovine Sp-1/chromogranin (BCG), cholecystokinin (CCK)-8, vasoactive intestinal polypeptide (VIP), gastrin, bombesin, secretin and pancreatic polypeptide (PP). Six kinds of endocrine cells were identified in this study. These immunoreactive cells were located in the gastric glands of stomach regions and in the basal portion of the epithelium of the intestinal tract or esophagus with variable frequencies. They were spherical or spindle-shaped. Serotonin-immunoreactive cells were observed in the whole alimentary tract including the esophagus. Somatostatin-immunoreactive cells were also detected throughout the alimentary tract except the rectum. CCK-8-immunoreactive cells were observed from the pylorus to ileum. VIP-immunoreactive cells were restricted to the rectum. Bombesin-immunoreactive cells were restricted to the fundic gastric regions and gastrin-immunoreactive cells were restricted to the pylorus. However, no BCG-, secretin and PP-immunoreactive cells were demonstrated in this study. In conclusion, the regional distribution and relative frequency of the endocrine cells in the alimentary tract of the tree frog were similar to other anuran species, but some differences which may be caused by feeding habits and species specification were also observed.

The tree frog, *Hyla arborea japonica*, belonging to the order Anura are widely distributed in Korea. Gastrointestinal endocrine cells which are dispersed in the epithelia and mucosal glands of the alimentary tract synthesized various kinds of gastrointestinal hormones and play an important role in the physiological functions of the alimentary tract (Bell, 1979). Until now, the investigation of gastrointestinal endocrine cells is considered to be an important part of the phylogenetic study (D'Este et al., 1994). In addition, the regional distributions and relative frequencies of these endocrine cells varied depending on the animal species and feeding habits (Solcia et al., 1975).

There has been a surge of interest in the endocrine cells of the alimentary tract in recent years. This is interesting considering the fact that so many gastroenteropancreatic (GEP) neuropeptides have been isolated

from amphibian skins (Nakajima et al., 1979; Van Noorden and Polak 1979). The GEP endocrine cells in various amphibians have been extensively studied by histochemical (Kim and Chung, 1973), electron microscopical (Gauze, 1971; Chung and Kwun, 1983) and immunohistochemical (Buchan, 1986) methods. Although about 17 types of endocrine cells including serotonin, somatostatin, glucagon, cholecystokinin (CCK)-8, chromogranin, pancreatic polypeptide (PP), bombesin, neurotensin, gastrin-releasing peptide, substance P, polypeptide YY, secretin, gastrin, vasoactive intestinal polypeptide (VIP), motilin, met-enkephalin,  $\beta$ -enkephalin and others have been detected in *Rana dybowskii* (Lee and Lee, 1996), *Rana pipens* (Lechago et al., 1978), *Xenopus laevis* (Lechago et al., 1978; Lee and Lee, 1992, 1997), *Rana esculenta* (Trandaburu and Nürnberger, 1995), *Bufo regularis* (El-Salhy et al., 1981, 1982), *Rana catesbeiana* (Lee et al., 1998a, 1999), *Rana nigromaculata* (Lee et al., 1998b) and 8 species of the anuran amphibian (Buchan, 1986), the endocrine cells of the tree frog have not been extensively

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studied except by Choi et al. (1999) who reported the localization of endocrine cells in the alimentary tracts of six frog species which inhabited Korea including the tree frog, *Hyla arborea japonica*. Their studies did not show any sufficient evidence namely, they used only six typed antisera (somatostatin, gastrin, CCK-8, PP, serotonin and glucagon). The present study aims to characterize the regional distributions and relative frequencies of endocrine cells in the alimentary tract of the tree frog, *Hyla arborea japonica*, which were investigated by immunohistochemical methods using specific antisera against serotonin, somatostatin, bovine Sp-1/chromogranin (BCG), CCK-8, VIP, gastrin, bombesin, secretin and PP.

**Materials and Methods**

Five adult tree frogs, *Hyla arborea japonica*, were captured in Kyungsan, Korea and used in this study. Samples from 6 portions of the alimentary tract (esophagus, fundic and pyloric portions of the stomach, duodenum, ileum and rectum) were fixed in Bouin's solution. After paraffin embedding, 3-4 μm serial sections were prepared by routine methods. Each representative sections were deparaffinized, rehydrated and immunostained by the peroxidase anti-peroxidase (PAP) method (Sternberger, 1979). Blocking of the nonspecific reaction was performed with normal goat serum prior to incubation with specific antisera (Table 1). After rinsing in phosphate buffered saline (PBS; 0.01 M, pH 7.4), the sections were incubated in secondary antiserum. They were then washed in PBS buffer and finally the PAP complex was prepared. The peroxidase reaction was carried out in a 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.01% H<sub>2</sub>O<sub>2</sub> in Tris-HCl buffer (0.05 M, pH 7.6). After immunostaining, the sections were lightly counterstained with Mayer's hematoxylin and the immunoreactive cells were observed under a light microscope.

**Results**

In this study, six kinds of immunoreactive cells were detected with antisera against serotonin, somatostatin, CCK-8, VIP, gastrin and bombesin. According to the

Table 1. Antisera used in this study

Antisera raised*	Code	Source	Diluton
Serotonin	BO68082C	BioGenex Lab., San Ramon	1 : 20
Somatostatin	PUO421295	BioGenex Lab., San Ramon.	1 : 20
BCG <sup>1</sup>	517210	Incstar Corp., Stillwater.	1 : 1,000
CCK-8 <sup>1</sup>	8643010	Immunonuclear Corp., Stillwater.	1 : 1,000
VIP <sup>1</sup>	B95 C	Sera Lab., Sussex.	1 : 1,000
Gastrin	PUO190796	BioGenex Lab., San Ramon.	1 : 20
Bombesin	8652015	Immunonuclear Corp., Stillwater	1 : 1,000
Secretin	BO67122A	BioGenex Lab., San Ramon.	1 : 20
PP <sup>1</sup>	PUO660495	BioGenex Lab., San Ramon.	1 : 20

\*All antisera were raised in rabbits.

<sup>1</sup> BCG: bovine Sp-1/chromogranin, CCK-8: cholecystokinin-8, VIP: vaso-active intestinal polypeptide, PP: pancreatic polypeptide

Table 2. Regional distributions and relative frequencies of the endocrine cells in the alimentary tract of the tree frog, *Hyla arborea japonica*

	Esophagus	Fundus of stomach	Pyloric portion of stomach	Duodenum	Ileum	Rectum
Serotonin	±*	++	+++	++	+	±
Somatostatin	±	±	++	+	±	-
BCG <sup>1</sup>	-	-	-	-	-	-
CCK-8 <sup>1</sup>	-	-	+	+	±	-
VIP <sup>1</sup>	-	-	-	-	-	+
Gastrin	-	-	+++	-	-	-
Bombesin	-	+	-	-	-	-
Secretin	-	-	-	-	-	-
PP <sup>1</sup>	-	-	-	-	-	-

<sup>1</sup> BCG: bovine Sp-1/chromogranin, CCK-8: cholecystokinin-8, VIP: vaso-active intestinal polypeptide, PP: pancreatic polypeptide

\*Relative frequencies; +++: numerous, ++: moderate, +: a few, ±: rare, -: not detected

regions of the alimentary tract, different distributions and relative frequencies of these immunoreactive cells were observed. These differences are shown in Table 2. However, no BCG-, secretin-, and PP-immunoreactive cells were detected in this study.

Spindle shaped serotonin-immunoreactive cells were demonstrated in the basal portions of the epithelia of the whole alimentary tract except for stomach regions where these cells were located in the gastric gland regions. A few serotonin-immunoreactive cells were detected in the esophagus, but a large or moderate numbers in stomach regions. They were observed in the duodenum with moderate frequencies, but decreased in the terminal portion of the large intestine (Fig. 1A-E).

Somatostatin-immunoreactive cells having spherical or spindle shapes were observed in the basal portion of the epithelia of the whole alimentary tract except for the rectum and stomach regions. In stomach regions, they resembled those of serotonin-immunoreactive cells but they were not found in the rectum. The relative frequencies of these immunoreactive cells were rare in the esophagus, the fundus and the ileum. In the pylorus, they were predominantly detected with moderate frequency but gradually decreased along the intestinal tract (Fig. 2A-E)

Spherical to spindle shaped CCK-8-immunoreactive cells were observed from the pyloric region to the ileum. These immunoreactive cells were located in the gastric glands of the pylorus in few frequency and in the basal portions of duodenum and ileum with a few or rare frequencies (Fig. 3A-C). Spindle shaped VIP-immunoreactive cells were demonstrated in the inter-epithelial regions of the rectum in few frequency (Fig. 4A).

Numerous gastrin-immunoreactive cells were restricted to the pylorus as spindle or spherical shapes. They were located in the gastric gland regions (Fig. 4B)

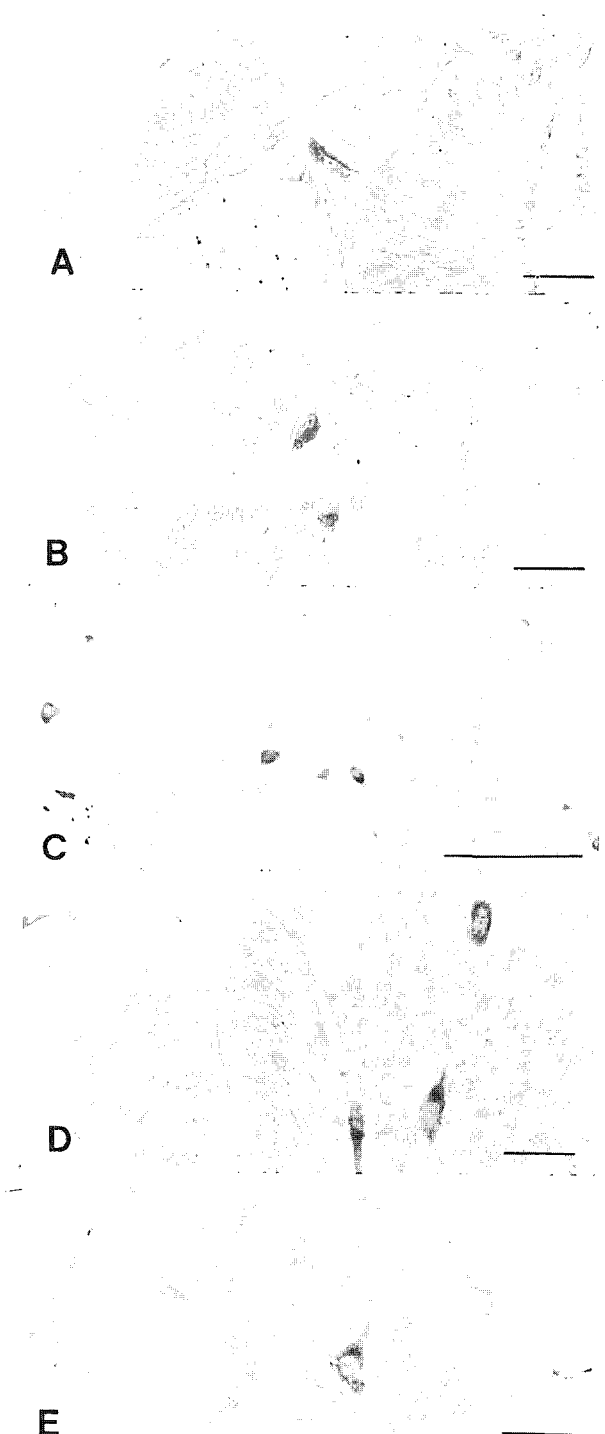
Bombesin-immunoreactive cells having spherical shapes were restricted to the basal portions of the gastric gland of the fundus in few frequency (Fig. 4C).



**Fig. 1.** Serotonin-immunoreactive cells in the esophagus (A), fundic (B) and pyloric portions of the stomach (C), duodenum (D) and ileum (E) of tree frog. Note that spherical or spindle shaped cells were observed in the interepithelial portion, basal portion of the epithelium or gastric gland regions. Scale bars=100  $\mu$ m.

### Discussion

Endocrine cells in the alimentary tracts appeared



**Fig. 2.** Somatostatin-immunoreactive cells in the esophagus (A), fundic (B) and pyloric portions of the stomach (C), duodenum (D) and ileum (E) of the tree frog. Scale bars=100  $\mu$ m.

remarkably different in the regional distribution, relative frequency and cell types according to the animal species and each regional part of the alimentary tract (Gabe, 1972; Alumets et al., 1977).



Fig. 3. CCK-8-immunoreactive cells in duodenum (A), pyloric region of the stomach (B) and ileum (C) of the tree frog. Scale bars=100  $\mu$ m.

Serotonin is composed of monoamines and widely distributed in the nervous system (El-Sahly et al., 1985). El-Sahly et al. (1985) reported that serotonin-immunoreactive cells were found throughout the gastrointestinal tract (GIT) of all species. The regional distributions and relative frequencies of serotonin-immunoreactive cells were detected in the whole GIT of *Rana dybowskii* (Lee and Lee, 1996), *Xenopus laevis* (Lee and Lee, 1992), *Rana nigromaculata* (Lee et al., 1998b) and *Rana catesbeiana* (Lee et al., 1999). In addition, Choi et al. (1999) reported the localization of serotonin-immunoreactive cells in the alimentary tracts of six frog species habited in Korea including the tree frog. According to these previous reports, these cells were most predominant in the pylorus except for *Rana nigromaculata* (Lee et al., 1998b) which were most predominant in the fundus and duodenum. It was reported that this variance of relative frequencies in the Anuran species might be due to sampling time or season (Lee and Lee, 1996). In the present study, these cells were observed in the whole alimentary tract as in previous reports but relative frequencies are somewhat different from other anuran species and the relative frequencies in the intestinal tract were quite

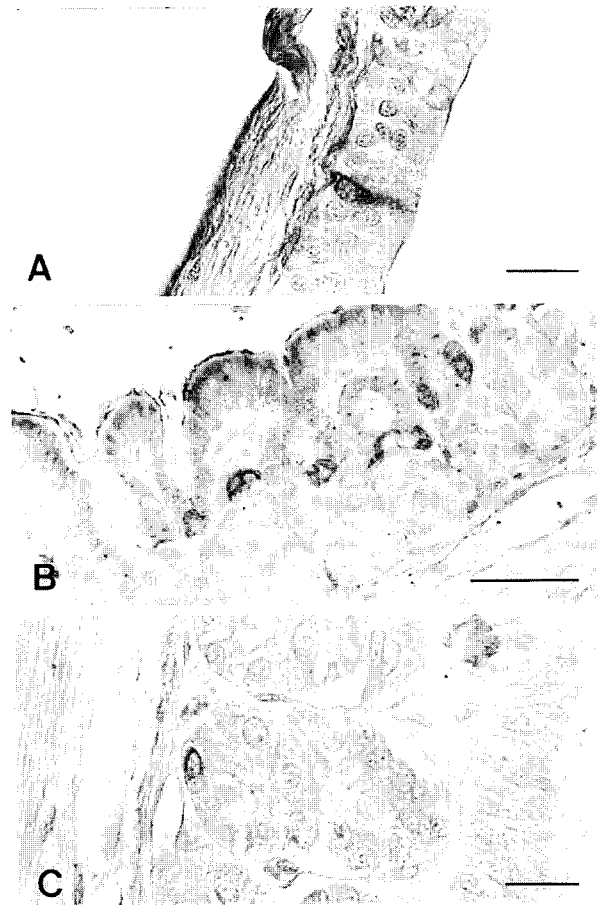


Fig 4. VIP-immunoreactive cells in rectum (A), gastrin-immunoreactive cells in the pyloric region of the stomach (B) and bombesin-immunoreactive cells in the fundus of the stomach (C) of the tree frog. Scale bars=100  $\mu$ m.

different from reported reported by Choi et al. (1999). Somatostatin, which consists of 14 amino acids, was first isolated from the hypothalamus of the sheep and it could be subdivided into a straight form and cyclic form (Brazeau et al., 1973). It is well known that somatostatin-immunoreactive cells show the widest distribution in the whole GIT of all vertebrate species investigated, including the primitive agnathans with serotonin-immunoreactive cells (Falkmer and Van Noorden, 1983). In the anuran species, these cells were detected in *Rana dybowskii* (Lee and Lee, 1996), *Rana esculenta* (Trandaburu and Nürnberger, 1995), *Xenopus laevis* (Lee and Lee, 1992), *Bufo regularis* (El-Sahly et al., 1981), *Rana nigromaculata* (Lee et al., 1998b), *Rana catesbeiana* (Lee et al., 1999), 8 species of Anura (Buchan, 1986) and six frog species which inhabit Korea including the tree frog (Choi et al., 1999). According to these reports, they were most predominant in the fundus but decreased distally along the GIT except for the tree frog which were most predominant in the pylorus and thereafter decreased toward the distal portion of the intestine and finally was not

detected in the rectum (Choi et al., 1999). In the present study, somatostatin-immunoreactive cells were detected throughout the whole alimentary tract except for the rectum and the regional distributions and relative frequencies agreed with those reported by Choi et al. (1999).

Chromogranins have been found to occur in a large variety of endocrine organs and cells outside the adrenal medulla, and they have been claimed as common markers of all neuroendocrine cells (Lloyd and Wilson, 1983; Cohn et al., 1984). However, chromogranin-immunoreactive cells were restricted to stomach regions in the *Xenopus laevis* (Lee and Lee, 1992), *Rana nigromaculata* (Lee et al., 1998b) and in this study. In addition, the relative frequencies in *Rana dybowskii* (Lee and Lee, 1996) were smaller than other typed endocrine cells. In addition, no BCG-immunoreactive cells were found in this study. According to these results, it is suggested that chromogranin was not suitable as a marker of other endocrine cells in anuran species.

It is generally accepted that gastrin and CCK-8 originated from the same ancestor and a large fraction of these cells found in the human duodenum, besides reacting with non-C terminal CCK antibodies and C-terminal gastrin/CCK antibodies, also show immunoreactivity with C-terminal gastrin-34 antibodies, colocalised with CCK in a variable portion of secretory granules (Solcia et al., 1989). In anuran species, gastrin-, CCK-8- or gastrin/CCK-immunoreactive cells were restricted to the pylorus, duodenum and ileum (Buchan, 1986; Lee and Lee, 1992, 1996; Choi et al., 1999). These distributions of CCK-8-immunoreactive cells in this study are consistent with previous reports but gastrin-immunoreactive cells were different from those of other anuran species restricted to the pylorus.

VIP is a 28 amino acid peptide, which was originally isolated from the porcine intestine and recognized for its potent vasodilatory effect (Said and Mutt, 1970). Immunoreactivity of VIP in the intestinal nerve was detected in seven species of the anuran species using immunohistochemical and radioimmunological techniques (Buchan et al., 1981). In this report, the regional distribution and relative frequency of VIP-immunoreactive cells showed species-specific differences and Larsson et al. (1979) reported that the distribution of VIP secretory endocrine cells demonstrated by different VIP antisera varied. In this study, spindle shaped VIP-immunoreactive cells not in the nerve fiber were found in the rectum in few frequency for the first in anuran species.

In the present study, bombesin-immunoreactive cells were restricted to the fundus. These findings are consistent with previous studies (Lechago et al., 1978; El-Sahly et al., 1981; Lee et al., 1998ab). Secretin-immunoreactive cells were not found in this study. These findings are similar to reports from Lee et al. (1998b), Buchan (1986), and Van Noorden and Falkmer

(1980) who demonstrated that these immunoreactive cells were not detected in lower vertebrates. However, in contrast to our results, El-Salhy et al. (1981) reported that secretin-immunoreactive cells were demonstrated in the whole GIT of *Bufo regularis* except for the rectum. It was reported that PP-immunoreactive cells were found in the stomach and small intestine of the anuran species (Falkmer and Stefan, 1978; El-Sahly et al., 1981; Choi et al., 1999). In contrast to these reports, PP-immunoreactive cells were not detected in this study.

In conclusion, the regional distributions and relative frequencies of the endocrine cells in the alimentary tract resembled other anuran species except for some differences which might be caused by feeding habits and species specification.

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