*J. Biol. Macromol.*, **2**(2), 76-77, 2002

**Note**

# Immunohistochemical study of the bcalization of glutamate decarboxylase in rodent's submandibular gland

Mineko Iwahori, Kanako Akamatsu, Sachie Kurohara, Kumio Yokoigawa, Hiroshi Ueno*, Hiroshi Ogawa,‡ Hitoshi Ozawa‡ and Mitsuhiro Kawata‡

Laboratory of Applied Microbiology and Biochemistry, Nara Women's University, Nara 630-8506, Japan and ‡Department of Anatomy and Neurobiology, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan

Received August 20, 2002; Accepted August 30, 2002

**Physiological role(s) of glutamate decarboxylase, a GABA-synthesizing enzyme, has been studied by using immunochemical method. In this study, we report that glutamate decarboxylase specifically localizes in submandibular gland of rat. Our results may suggest that glutamate decarboxylase plays a role in salivation.**

***Keywords***: GABA, glutamate decarboxylase, salivation, submandibular gland.

γ-Amino butyric acid (GABA),[1] synthesized by glutamate decarboxylase (GAD) from L-glutamic acid, is an inhibitory neurotransmitter in central nervous system (CNS) (*1*). We have shown that GAD activity is affected by various spice extracts *in vitro* (*2*). This earlier results have let us seek GAD proteins in the digestive system. If GAD proteins are found in the digestive system, it is highly likely that GAD is involved in some sort of regulation of food digestion via GABA synthesis.

It is of interests to consider which isozyme of GAD protein, GAD65 or GAD67, specifically localized in the digestive system, if there is any. Ever since the discovery of two isoforms of GAD (*3*), distinct roles of two forms have been suggested in various cellular events, for example, development, metabolism and homeostasis (*4*, *5*).

There was a report that GAD activity was observed in rats submandibular gland (*6*); however, little information was available for the localization and physiological functions of GAD in submandibular gland. In this study, we have examined rat submandibular gland by immunohistochemical staining to reveal the role of GAD in salivation.

## Materials and Methods

*Rats and Tissue preparation*

Wistar rats (9 weeks, Clea, Japan) were used for all of the reported studies. Experimental tissues were removed, dissected rapidly and fixed in 10% phosphate buffered formalin, pH7.4 for 24 h. After fixation, the samples were dehydrated through a graded ethanol series and embedded in Histosec (Merck Co., Darmastadt, Germany). Sections of 4 μm thickness were cut with a microtome, mounted on glass slides pre-coated with 3-aminopropyl triethoxysilane (Aldrich Chemical, Milwaukee), deparaffinized with xylene and rehydrated through a graded ethanol series, then washed for 5 min with running water and distilled water.

*Immunohistochemistry*

The sections were stained by diaminobenzidene (DAB) method using a commercial kit (Histofine SAB-PO(R), Nichirei Co., Japan) to allow visualization of GAD65 and 67. Slides were immersed in 3% $H_2O_2$ in phosphate buffered saline (PBS) for 7 min at room temperature to inhibit endogenous peroxidase activity. Additionally, we used a commercial kit (avidin-biotin endogenous blocking reagent set, Biomeda Co.) to block endogenous avidin and biotin. After washing with PBS, the sections were pre-incubated with 10% normal goat serum (Nichirei Co., Japan) for 30 min at room temperature in wet box, and then rinsed with PBS. The slides were incubated with primary antibody for GAD65/67 (Sigma-Aldrich, USA), GAD65 (Sigma-Aldrich, USA), or GAD67 (Alfa, USA) diluted 750, 2,000, 2500-fold, respectively, with PBS containing 1% bovine serum albumin (BSA) for 1 hr at root temperature in wet box. As negative controls, sections were incubated with PBS. After washing with PBS 3 times, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (Nichirei Co., Japan) for 30 min at room temperature in wet box. After 3 washes with PBS, the sections were incubated peroxidase-conjugated strepto-avidin (Nichirei Co., Japan) for 30 min at room temperature in wet box. The sections were washed twice for 5 min with PBS, and incubated with a commercial kit of chromagen DAB (DAKO Co.) at room temperature, then washed twice for 5 min with

*To whom correspondence should be addressed: Tel./Fax: 81-742-20-3493; E-mail: hueno@cc.nara-wu.ac.jp.

[1]The abbreviations used are: BSA, bovine serum albumin; CNS, central nervous system; DAB, diaminobenzidene; GABA, γ-amino butyric acid; GAD, glutamate decarboxylase; PBS, phosphate buffered saline.

distilled water. The sections were subsequently counterstained with methylgreen, followed by dehydration and mounted with a xylene-based mounting medium (entellan) and then observed under an optical microscope (BX50, Olympus, Tokyo, Japan).

## Results and Discussion

We have investigated immunolocalization of GADs in rat submandibular gland. When anti-GAD65/67 antibody (Ab) was employed, epidermal cells of ducts were stained, whereas acinar cells were not stained (Fig. 1a). To determine which isoform, GAD65 or GAD67, exists, we stained tissues with anti-GAD65 Ab and anti-GAD67 Ab (Fig. 1b and 1c). In the case using anti-GAD65 Ab, epidermal cells of ducts were strongly stained. While in the case using anti-GAD67 Ab, epidermal cells of ducts were also stained but only weakly.

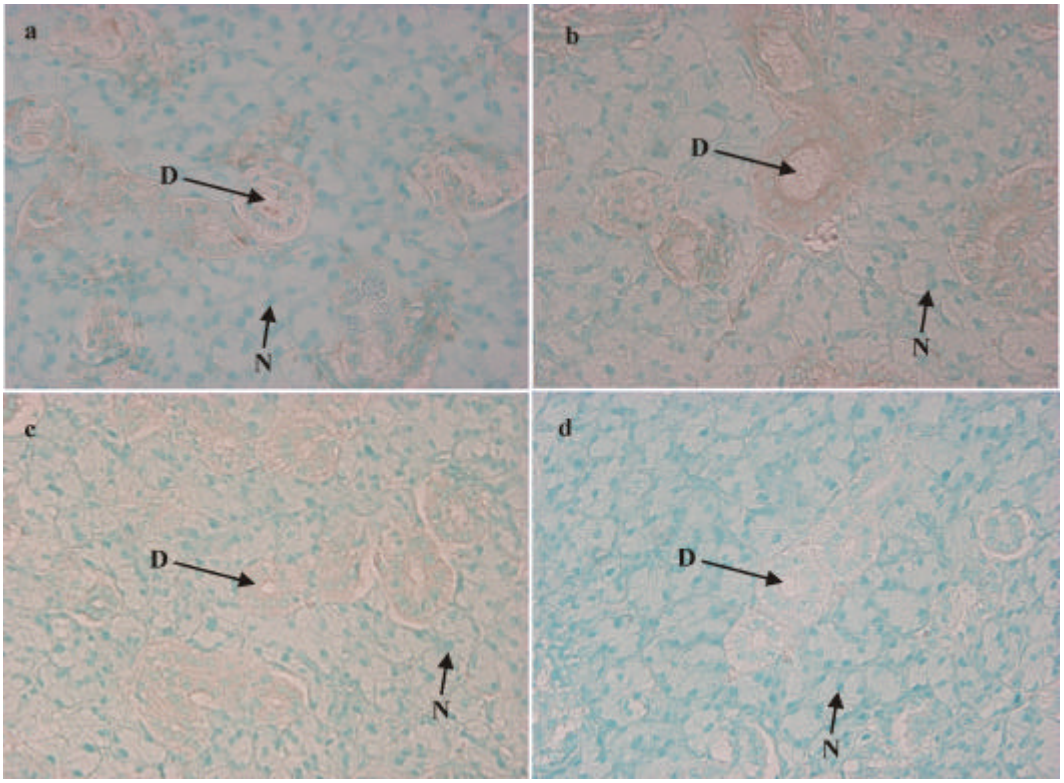


Fig. 1 Immunohistochemistry for GAD65 and/or GAD67 in rat submandibular gland. a: Stained with anti-GAD65/67 Ab. Locations of ducts (D) are marked in arrowheads. Nuclei (N) are stained with methylgreen. b: Stained with anti-GAD65 Ab. c: Stained with anti-GAD67 Ab. d: No staining with Ab. Photos were taken through video camera attached to the microscope having an objective lens of 40X.

In general, GAD65 is considered to localize in neurons and GAD67 is more widely distributed. However, our present results indicate both GAD isoforms being localized in ducts of submandibular gland; but, GAD65 appears to exist more than GAD67. It is of interests to investigate the role(s) of GAD65 in submandibular gland.

In rat's submandibular gland, $GABA_A$ receptor was found in acinar cells and striated ducts (*7*). It is also reported that GABA inhibits salivation by nerve stimulation. Therefore, it is probable to assume that salivation in rat is controlled by the action of GAD locating at the ducts of submandibular gland.

**Acknowledgements**

We would like to express sincere thanks to Drs. H. Miyamoto and N. Manabe of Kyoto University Graduate School of Agriculture for the contribution of their time and advice on this study. The skilful technical assistance of M. Yamaguchi-Yamada and S. Imanishi are also acknowledged.

**References**

1) Roberts, E. and Frankel, S., *J. Biol. Chem.* **188**, 789-795 (1951).
2) Kurohara, S., Asai, M., Hayashi, M., Yokoigawa, K., and Ueno, H., *J. Biol. Macromol.* **1**, 45-48 (2001).
3) Erlander, M.G., Tillakaratne, N.J.K., Feldblum, S., Patel, N., and Tobin, A.J., *Neuron* **7**, 91-100 (1991).
4) Dupuy, S.T. and Houser, C.R., *J. Neurosci.* **16**, 6919-6932. (1996).
5) Ueno, H., *J. Mol. Catalysis B: Enzymatic* **10**, 67-79 (2000).
6) Sawaki, K., Ouchi, K., Sato, T., and Kawaguchi, M., *Jpn J Pharmacol* **67**, 359-363. (1995).
7) Shida, T., Kondo, E., Ueda, Y., Takai, N., Yoshida, Y., Araki, T., Kiyama, H., and Tohyama, M., *Brain Res Mol Brain Res* **33**, 261-268. (1995).

Communicated by Sumio Ishijima