Micro-Enzyme Immunoassay of Vasoactive Intestinal Polypeptide (VIP)-like Immunoreactive Substance in Bovine Milk

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A sensitive and specific enzyme immunoassay for vasoactive intestinal polypeptide (VIP)-like immunoreactivity was developed with the use of synthetic carboxy-terminal (C-terminal) fragment (residue 11—28) of porcine VIP conjugated with β-D-galactosidase and a second antibody-coated immunoplate. Using 4-methylumbelliferyl β-D-galactopyranoside as a fluorogenic substrate, the minimum amount of VIP-like immunoreactive substance (VIP-IS) detectable by this method was 0.1 fmol/well (2.5 pmol/l). The level of VIP-IS in bovine foremilk was above 100 pmol/l, which was more than eightfold higher than that in normal milk.

Keywords vasoactive intestinal polypeptide (VIP)-like immunoreactive substance; bovine foremilk; VIP(11—28)-linked β-D-galactosidase; 4-methylumbelliferyl β-D-galactopyranoside; highly sensitive enzyme immunoassay

Vasoactive intestinal polypeptide (VIP) was first isolated from porcine small intestine by Said and Mutt.[1] This 28-amino-acid peptide has a wide range of actions on the cardiovascular system, respiratory system, digestive system, metabolism, endocrine system, central nervous system and immunosystem in mammals.[2] At present, the structures of porcine,[3] chicken,[4] bovine,[5] human,[6] dog,[7] guinea pig[8] and dogfish[9] VIPs are known (Fig. 1). We have succeeded in synthesizing porcine,[10] chicken[11] and guinea pig[12] VIPs.

Radioimmunoassay (RIA) of VIP has been developed by several authors.[13] However, in terms of safety, sensitivity and ease of handling, the existing methods are still less than satisfactory. We wish to report the development and application of an enzyme immunoassay (EIA) for VIP which circumvents the problems encountered with RIA. Using this EIA, we examined bovine milk and found that a large amount of VIP-like immunoreactive substance (VIP-IS) was present in bovine foremilk, compared to normal milk samples.

Experimental

Materials Bovine milk and foremilk were kindly supplied by Nakashibetsu Preparation Center, Mitsubishi Kasei Corp.

Synthetic porcine VIP, its fragments (positions 1—10, 11—28, 14—28, 18—28), porcine secretin, human glucose-dependent insulinotropic peptide (GIP), peptide histidine isoleucine (PHI) and human gastrin releasing peptide (GRP)[10,14] were used. Human atrial natriuretic peptide (ANP), substance-P (SP) and gastrin were purchased from Peptide Institute Inc. (Osaka, Japan) and bovine serum albumin (BSA), polyoxyethylene sorbitan (Tween-20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. β-D-Galactosidase (β-gal from *Escherichia coli*) and goat anti-rabbit immunoglobulin G (IgG) (TAGO 4120) were purchased from Boehringer Mannheim Corp. and TAGO Inc. (Burlingame, U.S.A.), respectively.

Antiserum to VIP (604/001) was purchased from UCB-Bioproducts S.A. and the lyophilized VIP-antiserum was reconstituted to 80 ml with the assay buffer (0.05 M phosphate buffer, pH 7.0, containing 0.5% BSA, 1 mM $MgCl_2$ and 250 KIU/ml aprotinin). All other chemicals were of analytical reagent grade.

Preparation of Milk Extract Bovine milk and foremilk were defatted by centrifugation at $10000 \times g$ at 4 °C for 30 min. Casein and other proteins were coagulated by acidification to pH 4 with 3% acetic acid (AcOH) and were removed by centrifugation. Defatted/de-caseinated milk samples were diluted threefold with 3% AcOH and were loaded on reversed-phase C_{18} cartridges (SEP-PAK, Waters Co. Inc., Milford, MA). After washing with 3% AcOH (10 ml), the VIP-ISs were eluted with 70% acetonitrile in 0.5% AcOH (2 ml). Eluates were concentrated by spin-vacuum evaporation and were subjected to EIA. Recovery of VIP by this extraction procedure was 94±6%.

Preparation of Enzyme-Labeled Antigen VIP(11—28) was conjugated onto β-gal by using EMC-succinimide according to the method of Kitagawa *et al.*[15] VIP(11—28) (0.99 mg) was dissolved in 0.05 M phosphate buffer, pH 7.0 (1 ml), and an aliquot of tetrahydrofuran (50 μl) containing EMC-succinimide (0.44 mg) was added. The mixture was stirred at 20 °C for 60 min, and then applied to a Sephadex G-25 column (1.5×51 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was eluted with the same buffer. Individual fractions (2.4 ml each)

H–His–Ser–Asp–Ala–⑤–Phe–Thr–Asp–⑨–Tyr–⑪–Arg–⑬–Arg–Lys–Gln–Met–Ala–⑲–Lys–Lys–Tyr–㉓–Asn–Ser–㉖–Leu–㉘–NH_2

	5	9	11	13	19	23	26	28
pig, cow								
human, dog	Val	Asn	Thr	Leu	Val	Leu	Ile	Asn
chicken	Val	Asn	Ser	Phe	Val	Leu	Val	Thr
guinea pig	Leu	Thr	Thr	Leu	Met	Leu	Val	Asn
dogfish	Val	Asn	Ser	Ile	Val	Ile	Leu	Ala

Fig. 1. Structure of Vasoactive Intestinal Polypeptide (VIP)

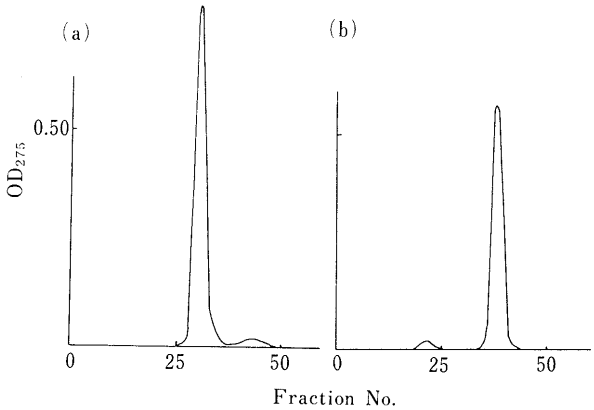

Fig. 2. Purification of VIP(11—28)-Linked β-D-Galactosidase

a, Gel-filtration of EMC-VIP(11—28) on Sephadex G-25: column, 1.5×51 cm; fraction, 2.4 ml; elution, pH 7.0, 0.05 M phosphate buffer.

b, Gel-filtration of VIP(11—28)-linked β-D-galactosidase on Sephacryl S-300: column, 1.5×57 cm; fraction, 2.4 ml; elution, pH 7.0, 0.05 M phosphate buffer containing $MgCl_2$ (0.1 mM).

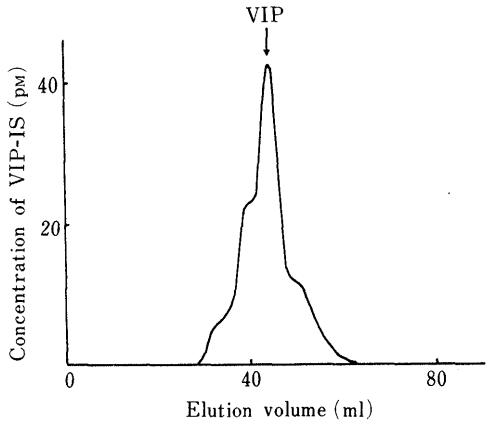

Fig. 3. Gel-Filtration of the Bovine Foremilk Extract on Sephadex G-25

Fractions were lyophilized and their content of immunoreactive VIP was measured. Synthetic VIP was run separately under the same conditions, and its elution volume is indicated by the arrow: column, 0.8 × 66 cm; elution, 0.6% AcOH.

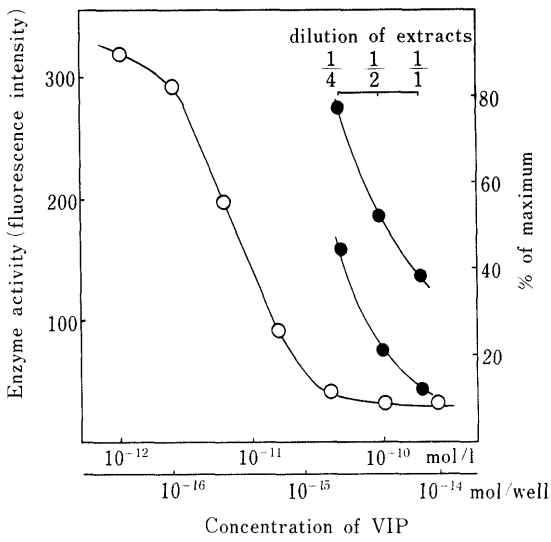

Fig. 4. Standard Curve of Synthetic VIP and Dilution Curves of Milk Extracts Obtained with Antiserum 604/001 by EIA

Synthetic VIP (○), milk extracts (●).

that showed absorbance at 275 nm were collected. The pooled EMC-VIP(11—28) fractions (No. 30—32 in Fig. 2a) were combined with β-gal (1.24 mg) by stirring at 20 °C for 60 min. The mixture containing VIP(11—28)-gal was applied to a Sephacryl S-300 column (1.5 × 57 cm) pre-equilibrated with pH 7.0, 0.05 M phosphate buffer containing 1 mM $MgCl_2$ and was eluted with the same buffer. Individual fractions (2.4 ml each, No. 20—23 in Fig. 2b) that showed absorbance at 275 nm were collected and stored at 4 °C after addition of 0.2% BSA and 0.1% NaN_3.

Preparation of Second-Antibody-Coated Immunoplates The washing buffer, pH 7.2, 0.01 M phosphate buffer containing 0.15 M NaCl and 0.05% Tween-20 and the coating buffer, pH 7.2, 0.05 M phosphate buffer containing 0.15 M NaCl, were used. Polystyrene plates (Nunc MicroWell Maxisorp F8 plate) were coated with the above coating buffer (100 μl/well) containing goat anti-rabbit IgG (10 μg/ml), then covered with Parafilm and kept at 4 °C overnight. The plates were washed 4 times with the above washing buffer. To block the plates, 200 μl of washing buffer containing 0.5% BSA was added to the wells and the plates were kept at 4 °C overnight. Then the plates were washed and stored at 4 °C until use.

Assay Procedure for VIP For assay, the above-mentioned assay buffer was used. Each sample (or standard) (100 μl) was incubated at 4 °C for 24 h with VIP-antiserum 604/001 (100 μl) in a test tube, and then the diluted enzyme-labeled antigen (50 μl) was added. The solution was further incubated at 4 °C for 24 h. Then, each antibody-antigen solution (100 μl) was added from the test tube to each well of the plate coated with anti-rabbit IgG mentioned above, and the plate was incubated at 4 °C overnight. The plate was washed 4 times with the washing buffer and 0.1 mM MUG (200 μl) in pH 7.0, 0.05 M phosphate buffer containing 1 mM $MgCl_2$ was added to each well of the plate. Then, the plate was incubated at 37 °C for 3 h and the resulting fluorescence intensities (emission at 450 nm and excitation at 360 nm) of each well were measured with a Corona MTP-100F microplate reader.

Gel-Filtration of Bovine Foremilk Extract A defatted bovine foremilk sample (1 ml) was shaken with 0.1% trifluoroacetic acid (TFA) in methanol (5 ml), then the solution was clarified by centrifugation (10000 × *g* for 30 min at 4 °C), and then supernatant was concentrated to dryness under vacuum. The residue was dissolved in 0.6% AcOH and the solution was applied to a Sephadex G-25 column (0.8 × 66 cm) pre-equilibrated with 0.6% AcOH. The column was eluted with the same solvent. The fractions (1.8 ml each) were lyophilized and submitted to EIA.

Results and Discussion

VIP is unstable, especially the amino-terminal (N-terminal) part. For example, peptide bond hydrolysis of the N-terminal part of VIP, resembling autolysis[16] and succinimide formation of the Asp–Asn sequence[10,17] have already been reported. Therefore, we selected the stable C-terminal VIP fragment, VIP(11—28), as a marker peptide and used β-gal-linked VIP(11—28) as a marker antigen.

Standard Curve A typical standard curve for the enzyme immunoassay of VIP is shown in Fig. 4. A linear displacement of binding with enzyme-labeled VIP(11—28) by synthetic VIP was obtained, when plotted as a semi-logarithmic function from 1.0 to 40 pM VIP. The minimum amount of VIP detectable by the present EIA system was 2.5 pM (0.1 fmol/well). An intraassay variation of 4% and interassay variation of 9% were obtained at 12 pM VIP. As also shown in Fig. 4, serial dilution of milk extracts gave a displacement curve essentially parallel to that of synthetic VIP.

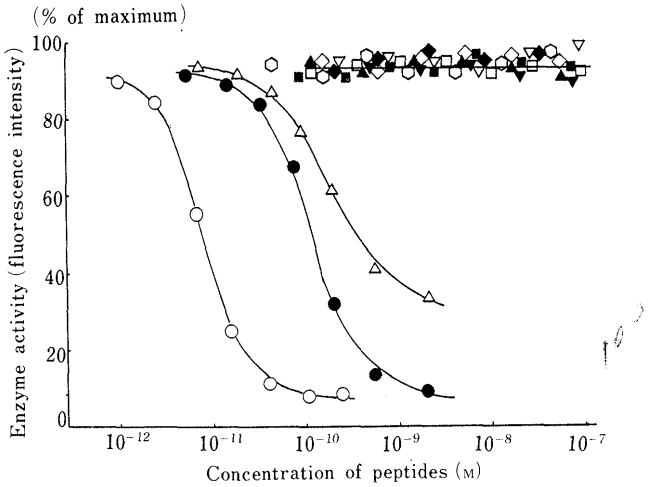

Fig. 5. Inhibition Curves of Various Peptides in EIA by Competition between VIP(11—28)-Linked β-D-Galactosidase and Various Peptides for Antiserum 604/001

VIP (○), VIP(11—28) (●), VIP(14—28) (△), VIP(18—28) (○), VIP(1—10) (▽), secretin (▼) PHI (□), GIP (■), gastrin (▼), GRP (◇), SP (◆).

Specificity of the Antiserum (604/001) Examined by EIA Immunospecificity of the antiserum (604/001) was examined by EIA using VIP(11—28)-gal. The displacement curves of various VIP-related peptides are shown in Fig. 5. VIP-(11—28) exhibited approximately 10% cross-reactivity as compared with synthetic VIP, and the displacement curve was parallel to that of VIP. VIP(14—28) inhibited the binding of VIP(11—28)-gal with the VIP-antiserum, but showed significantly reduced cross reactivity. VIP(18—28) and VIP(1—10) hardly inhibited the binding. Thus, it was

TABLE I. Level of VIP-like Immunoreactive Substance in Bovine Milk (pM)

Foremilk	139 ±32	$n=4$
Normal milk	15.9± 8.7	$n=10$

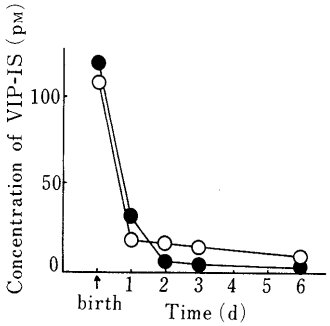

Fig. 6. Time Course of VIP-IS Levels in Bovine Milk Samples from Two Cows after Delivery

shown that the VIP-antiserum, 604/001, could recognize the central region of VIP. Secretin, PHI, GIP, gastrin, GRP and SP hardly inhibited the binding of VIP(11—28)–gal with the VIP-antiserum.

Measurement of VIP-IS in Milk Samples by EIA Concentrations of VIP-IS in bovine milk and foremilk were measured by EIA. The levels of VIP-IS in bovine foremilk were above 100 pM, while the levels of VIP-IS in normal bovine milk were much lower (Table I). The high levels of VIP-IS in foremilk dropped within 2 d after delivery (Fig. 6).

Gel-Filtration of Bovine Foremilk Extract on Sephadex G-25 Next, we examined the molecular variants of VIP-IS in a bovine foremilk sample. Fractionation of bovine foremilk extract on Sephadex G-25 revealed that the main VIP-IS were eluted at the same elution volume as synthetic VIP (Fig. 3).

Using β-galactosidase-linked VIP(11—28) as a marker antigen, anti-rabbit IgG coated immunoplates and 4-methylumbelliferyl β-D-galactopyranoside as a fluorogenic substrate, the present EIA allows detection of VIP at levels as low as 2.5 pM (0.1 fmol/well). VIP-IS in bovine milk was examined with this EIA system. The levels of VIP-IS in normal milk were 15.9±8.7 pM, which were in almost the same range as those detected by RIA reported by Werner *et al.*[18] However, the levels of VIP-IS in bovine foremilk were 139±32 pM, more than eightfold higher than that in normal milk, but dropped within 2 d after delivery (Fig. 6). The molecular size of VIP-IS in bovine foremilk was the same as that of VIP.

Milk is known to contain peptide hormones.[18-20] Moreover, the peptides retain their biological activities in milk[19,21] and when ingested by the neonate, appear intact in plasma.[22] VIP stimulates the secretion of insulin from newborn rat pancreas.[23] It also increases growth hormone release by inhibiting the action of somatostatin at the hypothalamic and pituitary levels.[24] Moreover, Acs *et al.*[25] reported that rat milk stimulated pituitary growth hormone secretion of neonatal pituitary. Furthermore, VIP stimulates androgen biosynthesis in cultured neonatal testicular cells.[26] Thus, VIP in bovine foremilk may have some growth-regulatory functions in newborn calves.

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