# Gonadotropin-Releasing Hormone-associated Peptide Immunoreactivity in Bovine Colostrum (43090)

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*Abstract.* **Gonadotropin-releasing hormone(GnRH)-associated peptide (GAP) is a 56-amino acid peptide found on the C-terminal of the GnRH (also called luteinizing hormone-releasing hormone) precursor and is assumed to be co-produced with GnRH. The purpose of this report is to demonstrate the presence of GAP immunoreactivity in bovine colostrum. Radioimmunoassay of acidified methanolic extracts demonstrated a concentration of GAP immunoreactivity of approximately 1.5 ± 0.1 pmol/g dry skim bovine colostrum. Gel filtration (Sephadex G-10) and high-performance liquid chromatography of extracts containing GAP immunoreactivity showed it to be of low molecular weight and a high hydrophobic character. The presence of GAP immunoreactivity in bovine colostrum suggests that the GnRH precursor is synthesized and processed in mammary tissue itself.** [P.S.E.B.M. 1990, Vol 194]

Gonadotropin-releasing hormone-associated peptide (GAP) is a 56-amino acid peptide and is located in the C-terminal of the GnRH precursor which was originally deduced from the cDNA of GnRH (1, 2). Radioimmunoassay and immunohistochemistry studies demonstrated the coexistence of GnRH and GAP immunoreactivity (GAP-IR) in mammalian and primate tissues (3–5). Earlier studies have also showed that GAP and its fragments can release luteinizing hormone (LH) and follicle-stimulating hormone (6, 7).

Recently, GnRH was found in the milk of the cow, rat, and human in high concentrations when compared with those in the general circulation and the hypothalamo-hypophyseal portal circulation (8–10). These findings implied a source of GnRH outside the central nervous system. The purpose of this study was to demonstrate the presence of GAP-IR in bovine colostrum by means of radioimmunoassay, gel filtration, and high-performance liquid chromatography.

## Materials and Methods

**Preparation of Mild Extract.** Dry skim bovine colostrum was provided by Hiya Pharmaceutical Co., Ltd. (Osaka, Japan). Since 1 g of skim bovine colostrum equals approximately 5 ml of original milk, skim milk powder was dissolved in a 5-fold volume of distilled water (5 ml/g), adjusted to pH 4.0 with HCl, and then centrifuged at 3000 rpm at 4°C for 30 min to remove casein. The supernatant was centrifuged again after adjusting the pH to 7.0 with concentrated $NH_4OH$ and then lyophilized. GAP-IR was extracted by reconstituting the lyophilized powder in 0.1 *N* HCl/90% MeOH (15 ml/g powder) and mixed at 4°C for 16 hr. This mixture was centrifuged and the supernatant was evaporated under reduced pressure. Distilled water was added (about 20 ml) and then the extracts were lyophilized. The recovery of $^{125}$I-hGAP(34–56) is 82 ± 3%.

**Radioimmunoassay (RIA).** An antiserum (R-2042) developed in our laboratory by immunizing rabbits with hGAP(34–56) conjugated to bovine serum albumin (BSA) was used to measure GAP-IR in this study. This antiserum recognizes the C-terminal region of hGAP (residues 34–40) and fails to cross-react with GnRH, somatostatin, and h-leumorphin (Fig. 1). 3-(4-Hydroxyphenyl)-propionyl (HPP)-hGAP(34–56) was iodinated by the chloramine-T method and hGAP(25–56) was used as a standard. RIA for GAP were done in a total volume of 0.7 ml of 0.01 *M* phosphate-buffered saline (0.025 *M* EDTA, 0.5% BSA, pH 7.4) containing 0.1 ml each of standard or sample, antiserum (final

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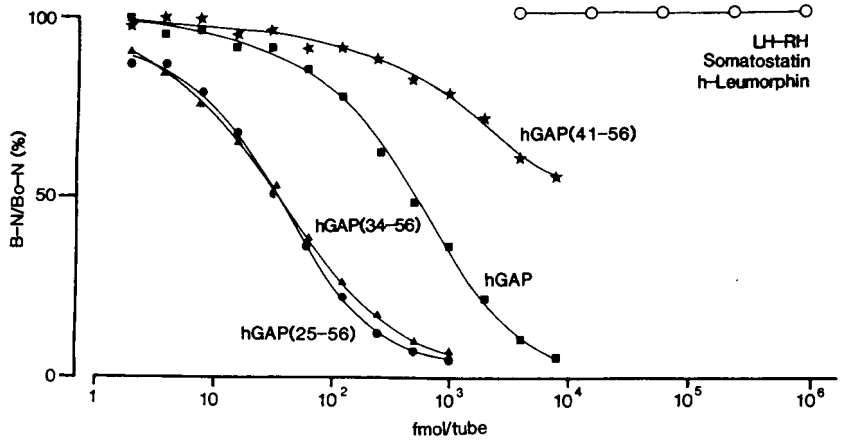


**Figure 1.** Characterization of anti-h-GAP(34–56) serum (R-0242). R-0242 was used at a final dilution 1/4900, and $^{125}$I-HPP-h-GAP(34–56) was used as tracer. The peptides used were h-GAP(1–56) (■), h-GAP(25–56) (●), h-GAP(34–56) (▲), h-GAP(41–56) (★), LHRH, somatostatin and h-leumorphin (○).

dilution 1/4900), and tracer (5000 cpm). After a 48-hr incubation at 4°C, free and bound ligands were separated by adding 0.1 ml of normal rabbit serum (dilution 1/150; Daiichi Radioisotope Labs., Ltd., Tokyo, Japan), 0.1 ml of goat anti-rabbit γ-globulin serum (dilution 1/30; Daiichi Radioisotope Labs., Ltd.), and 0.5 ml of 10% polyethylene glycol. After a 3-hr incubation at 4°C, this mixture was centrifuged at 3000 rpm for 30 min at 4°C and then aspirated. The precipitate was counted in a gamma counter. The sensitivity and $ID_{50}$ of the assay are 4 and 50 fmol/tube, respectively. The intra- and interassay coefficients of variation were 11% and 13%, respectively.

**Gel Filtration.** Sephadex G-50 superfine (1.6 × 100 cm) and Sephadex G-10 (1.0- × 100-cm) columns were used for gel filtration of the milk extracts using 1 *M* acetic acid and 3 *M* acetic acid containing 0.1% BSA as eluents, respectively. Extracts were dissolved in the respective solutions before chromatography. Fractions (1 or 2 ml) were collected, lyophilized, and then reconstituted in the RIA buffer for measurement of GAP-IR.

**High-Performance Liquid Chromatography (HPLC).** The GAP-immunoreactive component from the Sephadex G-10 fractionation was concentrated and injected onto a reverse phase HPLC system (Waters model-590) using a YMC-Pack R-ODS-5 column (4.6 × 250 mm) (Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan). Extracts were eluted with a linear gradient of 0–50% acetonitrile in 0.01 *N* HCl over 60 min at a flow rate of 1 ml/min. Fractions were collected at 1-min intervals, lyophilized to remove acetonitrile, and dissolved in the RIA buffer before measurement of GAP-IR.

## Results and Discussion

Figure 2 shows the displacement curves for the bovine colostrum extract and the GAP standard. The displacement curve for the colostrum extract is parallel to the displacement curve of the GAP standard, indicating that these substances are related structurally. This evidence suggests the existence of GAP-IR in bovine colostrum. It is worth noting that we used an antisera generated against a fragment of human GAP to characterize GAP-IR in the bovine colostrum extract. Bovine GAP, however, has not been sequenced yet. By RIA, GAP-IR was detected at a concentration of approximately 1.5 ± 0.1 pmol/g dry skim bovine colostrum.

Since the peak of GAP-IR eluted near the total volume position in our Sephadex G-50 studies, the extracts were subsequently applied to a Sephadex G-10 column equilibrated with 3 *M* acetic acid with 0.1% BSA. Gel filtration of the extract gave a single peak of GAP-IR with a $K_{av}$ of 0.5 (Fig. 3), indicating that GAP-IR in the milk extract is of low molecular weight. Furthermore, HPLC of this GAP-IR form obtained after Sephadex G-10 chromatography also showed only a single peak of GAP-IR with a long retention time (Fig. 4), suggesting that the GAP-IR in bovine colostrum has numerous hydrophobic amino acid residues.

The present report indicates the existence of GAP-IR in bovine colostrum. Since GnRH immunoreactivity has been found in bovine milk (0.08–2.54 pmol/ml) (8), our observation is compatible with earlier reports that GnRH and GAP are co-produced from the same precursor (3–5). Furthermore, the plasma levels of GnRH in the peripheral and hypophyseal portal circulation have been reported to be below 10 pg/ml (0.008 pmol/ml) and 30 pg/ml (0.024 pmol/ml), respectively (8, 11). One report showed a 5- to 6-fold higher level of GnRH in human milk when compared with that of plasma (9). These data suggest that the mammary tissue has an active concentrating mechanism for GnRH or that mammary tissue is an additional extrahypothalamic source for GnRH itself. Our data support the

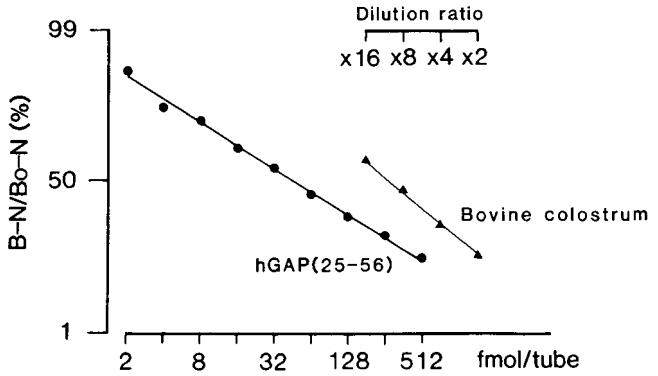


**Figure 2.** Dilution curves of hGAP(25–56) (●) and bovine colostrum extracts (▲) in RIA system using anti-hGAP(34–56) serum (R-0242) at a final dilution of 1/4900 and $^{125}$I-HPP-hGAP(34–56) as tracer.

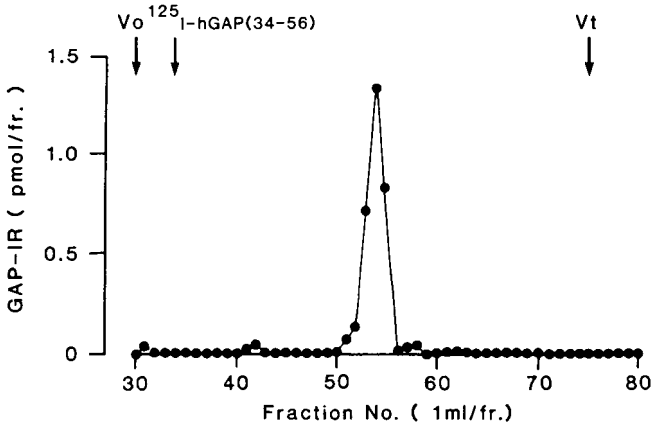


**Figure 3.** Gel filtration of bovine colostrum extracts. Extracts were chromatographed on Sephadex G-10 column (1.0 × 100 cm) eluted with 3 *M* acetic acid containing 0.1% BSA.

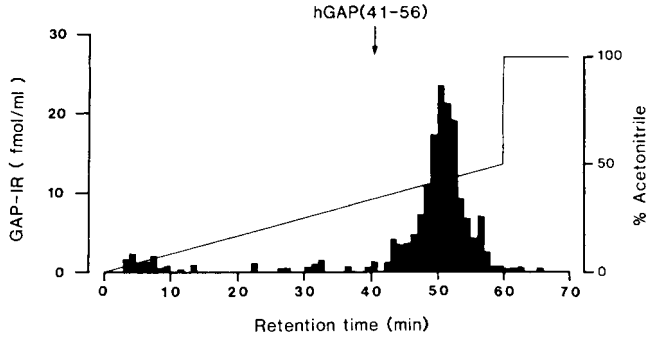


**Figure 4.** Reverse phase HPLC separation of GAP-immunoreactive component obtained from Sephadex G-10 chromatography. The sample was injected onto a HPLC system (Waters model 590) using YMC-Pack R-ODS-5 column (4.6 × 250 mm) with a linear gradient of 0–50% acetonitrile in 0.01 *N* HCl at flow rate of 1.0 ml/min.

second notion since a precursor associated fragment of GnRH is found in milk.

Since milk GnRH can stimulate gonadotropin release from the pituitary *in vitro* (8, 10, 12), it may explain the elevation of gonadotropins in sera of human and rat neonates (13, 14). A physiologic role of milk GnRH is suggested by the finding that rat LH serum levels are significantly lowered when neonates are prevented from suckling (8). In addition, one report indicates that the neonatal rat pituitary is not functionally linked to the hypothalamic source of GnRH (15). GAP can stimulate LH and follicle-stimulating hormone release from primary cultures of rat anterior pituitary cells and GAP (1–13) fragment had a stimulatory effect on cultured human and baboon anterior pituitary cells (6, 7). One report showed a co-secretion of GAP and GnRH into the hypophyseal portal circulation of sheep (16), but a gonadotropin-releasing effect of GAP and its fragments has not been found in sheep *in vivo* (17). Although speculative, the stimulatory effects of GAP and its fragments on LH and follicle-stimulating secretion may be species specific, or the peptide may exert its action only *in vitro*.

The present study shows only a low molecular weight form of GAP-IR in bovine colostrum. Since two forms of GAP-IR have been found in the rat hypothalamus (18), the GAP molecule may be further processed in mammary tissue or milk to only a single form which compared with the hypothalamus.

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