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PYY(1–36) is the major form of PYY in rat distal small intestine: quantification using high-resolution mass spectrometry

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Abstract

Here we measured molecular forms of PYY in the distal half of rat small intestine using a new method for tissue extraction, three sequential reverse phase chromatography steps, and PYY radioimmunoassay and mass spectrometry to measure their levels. The extraction method called RAPID, developed to minimize artifactual degradation of PYY during tissue extraction and sample preparation, uses **R**educed temperature, **A**cidified buffer, **P**eptidase inhibitors, **I**sotopically enriched mass spectrometry standards, and **D**ilution to inhibit and monitor endogenous peptide degradation during tissue processing. Synthetic peptides [PYY(1–36)-NH₂, PYY(3–36)-NH₂, PYY(1–36)-Gly-OH, and PYY(3–36)-Gly-OH] selectively enriched with ¹³C₃-alanine were added as internal standards to the extraction buffer. By collecting mass spectra rather than multiple-reaction-monitoring (MRM) profiles we simultaneously screen for any PYY forms that were present in the immunoreactive fractions. PYY(1–36)-NH₂, PYY(3–36)-NH₂, PYY(1–36)-Gly-OH, and PYY(3–36)-Gly-OH were identified and quantified at 64.3 ± 4.5, 6.1 ± 0.9, 0.9 ± 0.1, and <0.3 pmol/g of tissue, respectively (n=3). Thus, we found that in rat distal small intestine proPYY is processed to PYY(1–36)-NH₂ with little conversion to PYY(3–36)-NH₂. These data suggest that production of PYY(3–36)-NH₂ (a form with greater potency than PYY(1–36)-NH₂ for inhibition of feeding and gastric emptying) occurs after the peptide leaves its cell of synthesis by enzymatic action in the circulation.

INTRODUCTION

The 36 amino acid gut hormone peptide YY (1–36)-amide [PYY(1–36)-NH₂] was first discovered using an assay to detect peptides with carboxyl-terminal amides in porcine intestinal extracts [1, 2]. Peripheral administration of PYY(1–36)-NH₂ decreases pancreatic

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secretion [1, 3], gastric emptying [4, 5], gastric acid secretion [6–8], blood glucose [9] and intestinal motility [10]. In 1989, our group discovered a new molecular form of PYY, PYY(3–36)-NH₂ [11]. In 2002, Batterham *et al.* reported that PYY(3–36)-NH₂ inhibits food intake in humans and rodents [12]. Numerous groups have since confirmed that PYY(3–36)-NH₂ reduces food intake in several species including rodents, monkeys, and humans [5, 13–24]. Furthermore, PYY(3–36)-NH₂ is more potent than PYY(1–36)-NH₂ in reducing food intake and gastric emptying in rats [4, 15] and humans [5, 23]. In addition, our laboratory has recently observed the presence of glycine-extended forms of PYY in canine intestinal tissue [25].

Thus, three different molecular forms of PYY have been described and the physiological significance of these forms depends on their relative potencies and *in vivo* concentrations. However, reliable measurements are lacking for the *in vivo* concentrations of these peptide forms. Therefore, defining the physiological roles of the *in vivo* forms of PYY requires development of protocols to accurately identify and independently measure each form in tissue and blood with proper controls for *ex vivo* processing activity.

The proposed processing of proPYY to PYY(3–36)-NH₂ is as follows: i) the amino terminus of PYY is formed as it enters the endoplasmic reticulum by the action of signal peptidase; ii) the carboxyl terminus of PYY(1–36)-Gly-OH and PYY(1–36)-NH₂ is formed in sequential steps in the golgi and secretory vesicle by the actions of prohormone convertase [26], carboxypeptidase E [27], and peptidylglycine- α -amidating monooxygenase (PAM) [28], iii) dipeptidyl peptidase-IV (DPP-IV) converts PYY(1–36)-Gly-OH to PYY(3–36)-Gly-OH and PYY(1–36)-NH₂ to PYY(3–36)-NH₂. The relative proportions and bioactivities of the various intermediate forms of PYY [PYY(1–36)-NH₂, PYY(3–36)-NH₂, PYY(1–36)-Gly-OH, PYY(3–36)-Gly-OH] in tissue and blood have not been clearly determined.

Characterization of PYY in gastrointestinal tissue began in the 1980's with the development of PYY-specific radioimmunoassays (RIA) [29–31]. These studies did not use chromatographic methods to separate the different molecular forms of PYY. Our laboratory used PYY RIA in combination with high pressure liquid chromatography (HPLC) to separate and independently quantify PYY(1–36)-NH₂ and PYY(3–36)-NH₂ in human colon [11] and blood [32], and in rabbit [33] and dog colon [34]. In each study, PYY(1–36)-NH₂ and PYY(3–36)-NH₂ were present in roughly equal amounts. These data provided strong evidence that significant conversion of PYY(1–36)-NH₂ to PYY(3–36)-NH₂ occurs within the intestinal cells before secretion of the peptides. However, a limitation of these studies was that they did not use internal standards to monitor recovery and modification of endogenous PYY forms during extraction and purification from tissue and blood.

We recently developed the RAPID (**R**educed temperature, **A**cidified, **P**eptidase inhibited, **I**sotopically-enriched mass spectrometry standards and **D**iluted) method for extracting and purifying peptides from tissue [25, 35]. This method minimizes *ex-vivo* enzymatic and chemical breakdown of peptides and uses internal standards to monitor their recovery during extraction and purification. Using this method and high-resolution mass spectrometry to quantify the PYY forms, we determined that PYY(1–36)-NH₂ and PYY(3–36)-NH₂ account for 79 and 5%, respectively, of total PYY in canine ileum [25].

The higher PYY(3–36)-NH₂ levels observed in earlier studies suggest that significant *ex vivo* conversion of PYY(1–36)-NH₂ to PYY(3–36)-NH₂ may have occurred during tissue and blood processing. Here we measured PYY molecular forms in rat lower small intestine using the RAPID method to prepare samples for quantification using high-resolution mass spectrometry. This method can accommodate complex protein isoforms [37, 38] and

simultaneously look for degradation or enzymatic processing products occurring after sampling and prior to assay of the forms and levels present in the blood or tissue.

EXPERIMENTAL PROCEDURES

Peptides

Rat [$^{13}\text{C}_3$ -Ala^{3,7,12,22}]-PYY analogs ($^{13}\text{C}_{12}$ -PYY) (Table 1) were synthesized in the City of Hope Peptide synthesis facility using 9-fluorenylmethoxycarbonyl (Fmoc) strategy and Fmoc- $^{13}\text{C}_3$ -Alanine (purchased from Sigma, St. Louis, MO) as described previously (36). Rat, dog and pig PYY share the same sequence while human PYY differs at two amino acids (human 3I→rat 3A and 18N→18S, [32]). Synthetic peptides were purified by reverse phase HPLC on a C18 column with acetonitrile (ACN) elution gradients. The identities of the purified peptides were verified by mass and sequence using an electrospray ionization source coupled to a tandem mass spectrometer. Peptide purity was evaluated by reverse phase HPLC and mass spectral analysis and was above 95%. The concentrations of the final stock solutions were determined by UV absorbance at 280 nm using the Beer-Lambert law. All other peptides were obtained from the CURE/UCLA peptide synthesis facility.

Radiolabeling of PYY

Synthetic rat PYY(1–36)-NH₂ in sodium phosphate buffer (0.2 M, pH 7.4, 10ug in 20 ul) was treated with Na¹²⁵I (500 μCi in 5 μL NaOH solution pH 10, MP Biomedicals, Irvine CA). Chloramine T (10 μg in 10 μL of sodium phosphate buffer at pH 7.4) was added, and after 20 seconds the oxidation reaction was quenched by addition of an equal volume of 50% acetic acid. The labeled peptide was separated from the free ¹²⁵I by G-10 gel-permeation chromatography (Sephadex, Pharmacia, Uppsala, Sweden). The early eluting radioactive material was pooled, diluted 3-fold with an aqueous solution of 0.1% trifluoroacetic acid (TFA) and loaded onto an analytical reverse phase column (4.6 × 250 mm, C18, Vydac 218TP54, Hesperia, CA) and eluted with a 20% to 50% ACN gradient over 60 minutes. The latest major HPLC peak of counts per minute (CPM) eluting at approximately 38% ACN was well separated from the un-reacted peptide that eluted at approximately 40% ACN. This purified ¹²⁵I-PYY(1–36)-NH₂ was used in the recovery studies and in the RIA. ¹²⁵I-PYY(1–36)-NH₂ was stable for up to two months when stored at –80°C.

Tissue extraction

Male Sprague-Dawley (Charles River Laboratories, Wilmington, MA) 150 to 250 gram freely fed rats were used with the approval of the Veterans Administration of the Greater Los Angeles Healthcare System animal committee. We chose to extract ileal tissue as it was shown to contain the highest levels of PYY in one study [39] and it is the region where nutrients first encounter major levels of PYY producing cells as they pass through the gastrointestinal tract. Immediately after sacrifice, the peritoneal cavity was exposed; the end of the duodenum at the pylorus to the end of the ileum at the cecum was elongated and cut in half. The lower half was rinsed with cold saline and frozen on dry ice as rapidly as possible. The tissue was then placed in a minus 80°C freezer until used for extraction.

A modification of the RAPID method previously developed for isolation of peptides from dog tissue was used [25]. Frozen portions of the distal lower intestine were weighed (*ca.* 7 g), pulverized using a mortar and pestle under liquid nitrogen, diluted 1:12 (w/v, *ca.* 84 mL) in ice-cold extraction buffer containing 10% acetic acid, 10% ACN, 10,000 CPM ¹²⁵I-labeled PYY(1–36)-NH₂, 1200 pmol $^{13}\text{C}_{12}$ -PYY(1–36)-NH₂, 400 pmol $^{13}\text{C}_{12}$ -PYY(3–36)-NH₂, 200 pmol $^{13}\text{C}_{12}$ -PYY(1–36)-Gly-OH, 200 pmol $^{13}\text{C}_{12}$ -PYY(3–36)-Gly-OH and 1 μg/mL each of the following protease inhibitors from Peptides International (Louisville, KY):

diprotin A [DPP-IV inhibitor], E64D (broad spectrum cysteine endopeptidase inhibitor), aprotinin (broad spectrum serine protease inhibitor), Ac-SIMP-1 (matrix metalloendopeptidase inhibitor), and antipain (broad spectrum serine protease and cysteine endopeptidase inhibitor). In one separate control experiment to check for residual enzymatic activity the only isotopically enriched standard added to the extraction buffer was 1200 pmol of $^{13}\text{C}_{12}$ -PYY(1–36)-Gly-OH.

The slurry was shaken and then centrifuged (30 minutes, 4°C, 3000g). The supernatant was split into two equal aliquots and each portion loaded onto a disposable reverse phase cartridge (C18, 10 g, SepPak®, Waters, Milford, MA) which had been previously activated with ACN/water/TFA (10 mL, 90/10/0.1, v/v/v) and then equilibrated with 25 mL of 0.1% TFA/water. After loading the supernatant the SepPak was washed with 0.1% TFA/water until the absorbance of the eluate at 220 nm decreased to baseline (approximately 80 mL). The SepPak was then eluted with ACN/water/TFA (40 mL, 80/20/0.1, v/v/v). The radioactivity in the resulting fractions (4 mL) was determined with a gamma counter for the presence of ^{125}I -PYY(1–36)-NH₂. Radioactivity-containing fractions were pooled, diluted eight-fold with ACN/water/TFA (10/80/0.1, v/v/v) and loaded onto a semi-preparative reverse phase column (C4, 10 × 250 mm, Vydac 214TP510) at 4 mL/minute. We have previously shown that all PYY immunoreactivity elutes with the label during the SepPak chromatography under these conditions [25]. In addition, aliquots were taken for RIA with CURE PYY antibody 9153. The C4 column was eluted (2 mL/min) with a linear gradient from 10% to 90% ACN in 180 minutes. Fractions (1 mL) were collected and counted with the gamma counter for the presence of ^{125}I -PYY(1–36)-NH₂, and aliquots were taken for PYY RIA. The SepPak and C4 fractions were assayed for PYY-like immunoreactivity (PYY-LI) within 12 hours of collection. PYY-LI containing fractions eluting near the peak of radioactivity were pooled and loaded onto an analytical phenyl column (4.6 × 250 mm, Vydac 219TP54) that was eluted (1 mL/min) with a linear gradient of 20% to 30% ACN over 100 minutes with fractions collected every 1 min. This column and gradient separates PYY(3–36)-NH₂ and PYY(1–36)-NH₂ by about 3 min. Aliquots taken for RIA identified fractions containing PYY-LI in the position expected for PYY(3–36)-NH₂ and PYY(1–36)-NH₂. These fractions were pooled and partially dried down to remove ACN before mass spectral analysis.

PYY Radioimmunoassay

Standard curves were made with PYY(1–36)-NH₂ solutions with concentrations calculated from their absorbance at 280 nm. CURE antiserum 9153, used at a 1:50,000 dilution, reacts equally well with PYY(1–36)-NH₂ and PYY(3–36)-NH₂. The label used was ^{125}I -PYY(1–36)-NH₂. Aliquots from SepPak and HPLC fractions (20 µL, diluted 10-fold when necessary), with standard curve peptides, were diluted to 600 µL with RIA buffer (0.1 M sodium phosphate buffer, pH 7.5, containing 0.05 M NaCl and 0.025 M disodium ethylenediaminetetracetic acid, 0.1% w/v RIA grade bovine serum albumin and 0.1% Triton X-100, Sigma Chemical Co., St. Louis MO). The sample was vortexed (10 sec) and after 30 min at room temperature was diluted with antisera in RIA buffer (200 µL) and approximately 5000 cpm of labeled PYY in RIA buffer (200 µL). After incubation (16 h, 4°C) the free and bound radiolabels were separated with charcoal previously equilibrated with Dextran-70 for 16 h. Standard curves were prepared by plotting the Bound cpm/Free cpm (B/F) percentages after non-specific binding blank correction using a Creative Research Immunoassay computer program. The PYY-LI content of samples was calculated by interpolation from B/F percentage with standard curves to PYY(1–36)-NH₂.

Mass spectrometry

Analysis of peptides by nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS)

Samples were analyzed by nLC-ESIMS with high-resolution mass spectrometry performed on a hybrid linear ion-trap 7 Tesla FT-ICR mass spectrometer (LTQ-FT Ultra, Thermo Fisher Corporation, San Jose, CA) fitted with the manufacturers' nanospray source. After drying to approximately 50 μL , 5 μL aliquots of each sample was loaded onto a previously equilibrated reverse-phase trap (C18; Microtech, Vista, CA) at 2 $\mu\text{L}/\text{minute}$ in buffer A (0.1 % formic acid, 5 % ACN) and washed for 10 minutes with the same buffer. Flow was then switched to a reverse-phase column (75 $\mu\text{m} \times 10 \text{ cm}$; C18, 5 μm , 300 $\text{\AA}$; MicroTech) previously equilibrated for 20 minutes at 0.3 nL/min with 100 % eluant A. The trap was eluted onto the analytical column with a compound linear gradient (min/% 0.1% formic acid in ACN (eluant B); 0/0, 10/0, 8/20, 13/35, 23/75, 23.1/90). Column eluent was directed to a stainless steel nano-electrospray emitter (ES301; Proxeon, Odense, Denmark) at 2.4 kV for ionization without nebulizer gas. The m/z resolving power of the instrument was set at 100,000 (defined by $m/\delta m_{50\%}$ at m/z 400) allowing a mass spectrum to be recorded once per second. The first set of experiments was performed in full scan mode (350–2000 m/z) while a second set was performed in narrow scan mode (846.50–876.50 m/z) to maximize intensity of low abundance species. Mass spectra were analyzed with Qualbrowser software (Thermo Fisher).

RESULTS

Tissue extraction

Consistent with our previous experience, the majority of the immunoreactive forms of PYY co-elute in the SepPak and C4 semi-prep HPLC steps (Figure 2). The phenyl column and acetonitrile gradient separates PYY(3–36)-NH₂ from the co-eluting PYY(1–36)-NH₂ and PYY(1–36)-Gly-OH forms (Figure 3). The phenyl HPLC peaks were tentatively assigned as PYY(3–36)-NH₂ (60 min.) and PYY(1–36)-NH₂/PYY(1–36)-Gly-OH (63 min.) by elution position relative to each other and relative to standard and radiolabeled peptides. The peak assignments were confirmed by mass spectrometry and the identities of the various forms were established by comparison of calculated and observed monoisotopic masses (Table 2). The calculated and observed masses differed by less than 2 ppm.

The total PYY immunoreactivity was used to track PYY forms through the purification protocol. The ratio of the peak heights in the mass spectra between the matched ¹³C-enriched peptide standards and the endogenous peptides were used to calculate the amount of endogenous peptide present in the samples. Three separate RAPID extractions were performed, and in each the same pairs of pentuply-charged ions (the most intense signals in the spectra) were used to calculate concentrations of endogenous peptides. In addition, for each MS data set a minimum of three and up to six standard and endogenous peptide pair intensity ratios were calculated for each PYY form and adjusted by the initial standard concentration to obtain the amount of the endogenous PYY form present in the sample. The more abundant endogenous forms [PYY(1–36)-NH₂ and PYY(3–36)-NH₂] were measured in the full scan (350–2000 m/z) mode (Figure 4A), while PYY(1–36)-Gly-OH was measured using a second LC-MS run on the same fractions in which a narrower mass range (m/z 846.50–876.50) was scanned in order to improve the signal to noise ratio for low abundance species (Figure 4B).

The benefits afforded by multiple reaction monitoring (MRM) experiments for quantification of pre-selected species were outweighed by the advantages of being able to inspect full mass spectra for the presence of other PYY isoforms. Collecting full scan data allows all forms of PYY to be assessed simultaneously in the immunoreactive fractions.

Thus if there is conversion of the [$^{13}\text{C}_{12}$ -PYY(1–36)-Gly-OH] standard to a processed form due to enzymatic activity or chemical breakdown this would be apparent in the full scan data. For example, PAM or DPP-IV action on the $^{13}\text{C}_{12}$ -labeled standards would be apparent by m/z signals corresponding to $^{13}\text{C}_{12}$ -labeled amidated or 3–36 forms of PYY in the mass spectra. If the measurements were focused on one form at a time using selective ion monitoring this information would be lost.

Using this approach PYY(1–36)-NH₂, PYY(3–36)-NH₂, and PYY(1–36)-Gly-OH were measured at 64.3 ± 4.5 , 6.1 ± 0.9 , and 0.9 ± 0.1 pmol/gram of tissue, respectively, (n=3 extractions) in the lower half of the rat small intestine. In addition, no detectable signals were observed for any other forms predicted forms of PYY, including PYY(3–36)-Gly-OH. The estimated level of detection in this assay is around 0.3 pmol/g. These data indicate PYY(1–36)-NH₂ to be 90%, PYY(3–36)-NH₂ to be 9% and PYY(1–36)-Gly-OH to be 1% of total PYY in the lower half of the rat small intestine (Table 3). The low signal to noise data available for quantification of PYY(1–36)-Gly-OH could result in a slight underestimate of abundance when measured in this way (34; 35). PYY(3–36)-Gly-OH was undetectable in these experiments.

In a single control experiment, $^{13}\text{C}_{12}$ -PYY(1–36)-Gly-OH was added to the extraction buffer to determine whether proteases, like DPP-IV and PAM, produced an *ex vivo* conversion of PYY forms during extraction and purification. The results showed (*vide MS*) that less than 1% of the standard was converted to $^{13}\text{C}_{12}$ -PYY(3–36)-Gly-OH, $^{13}\text{C}_{12}$ -PYY(1–36)-NH₂, or $^{13}\text{C}_{12}$ -PYY(3–36)-NH₂, indicating the RAPID method effectively minimized artifactual conversion of the standards during tissue extraction and sample processing.

Discussion

Our results show that rat lower small intestine contains PYY(1–36)-NH₂, PYY(3–36)-NH₂, PYY(1–36)-Gly-OH, and PYY(3–36)-Gly-OH at 64, 6, 1, and <0.3 pmol/g of tissue, respectively. Thus, PYY(1–36)-NH₂, PYY(3–36)-NH₂, and PYY(1–36)-Gly-OH account for 90, 9, and 1%, respectively, of total PYY in rat lower small intestine. The total amount of ileal PYY immunoreactivity observed here, 71 pmol/g tissue, is significantly higher than that reported by Gomez *et al.* for ileal tissue ([40] 17 pmole/g tissue). This difference may be due to the extraction methods employed. Greeley *et al.* used boiling of frozen tissue in 0.5 M acetic acid while we used the RAPID method developed to optimize peptide recovery and stability. The postulated reasons for differences between our results and those of Greeley have not been tested. However, the present results for the levels of PYY in the ileum were more similar to those found by Aponte *et al.* [39] (84 pmole/g tissue) who utilized an acid ethanol extraction method. In the work of Aponte *et al.*, the highest levels of PYY immunoreactivity was found in rat ileum rather than colon. The various forms of PYY were not studied in these early papers [39, 40].

The results observed here differ significantly from those observed in our earlier work on PYY forms in man, dog and rabbit colonic mucosa ([11, 33, 34]), which used more conventional extraction techniques with no external monitoring of *ex-vivo* modification of extracted peptides. Here we examine the forms and levels of PYY present with the RAPID method and suggest that the relatively larger amounts of PYY(3–36)-NH₂ observed in earlier studies could be due to significant *ex vivo* conversion of PYY(1–36)-NH₂ to PYY(3–36)-NH₂ during tissue extraction and peptide purification. However we cannot exclude the possibility that different sections of the intestine produce different proportions of PYY(3–36)-NH₂.

In addition we detected glycine-extended PYY(1–36) in this study and in our earlier canine tissue study where this form had not been previously observed [25]. This is important because glycine extended forms of gastrointestinal peptides have been reported to have physiological relevance ([25] and references therein). However, the low levels of glycine-extended PYY in the rat ileum (~1%) and the lack of binding to Y-receptor subtypes (data not shown) suggests that PYY-Gly is not an important source of PYY physiological activity. However, levels of PYY-Gly in colon and other sections of the intestine and in other species should be examined to determine if this peptide is more prevalent in other areas and thus of greater physiological relevance.

In this work we show that PYY(3–36)-NH₂ is a minor form of rat tissue PYY at the site of the initial release of PYY with passage of nutrients through the intestine. Similarly, in our canine tissue study, PYY(1–36)-NH₂, PYY(3–36)-NH₂, and PYY(1–36)-Gly-OH were estimated to be 79, 5, and 16%, respectively, of total PYY [25]. Thus, both studies showed significantly higher levels of PYY(1–36)-NH₂ than PYY(3–36)-NH₂ in tissue from the distal small intestine, yet higher levels of PYY(1–36)-Gly-OH in dog compared to rat.

Our results support the scheme shown in Figure 1 for processing of proPYY. In this scheme the amino terminus of PYY is formed as it enters the endoplasmic reticulum by the action of signal peptidase. The carboxyl terminus of PYY(1–36) is formed in sequential steps by the actions of prohormone convertase [26], carboxypeptidase E [27], and peptidylglycine- α -amidating monooxygenase (PAM) [28]. Most if not all of the PYY(1–36)-NH₂ remains intact in secretory granules. The degree to which PYY(1–36)-NH₂ is converted to PYY(3–36)-NH₂ by DPP-IV following secretion and whether PYY processing is similar in species other than rat and dog, and in tissues other than the small intestine where PYY is also produced (*e.g.* colon and brain) remains to be determined.

These observations are physiologically relevant because the formation of the more potent form of PYY (PYY(3–36)-NH₂) for inhibition of food intake and gastric emptying occurs after secretion of PYY(1–36) from the L-cell. Without the action of DPP-IV in the circulation the physiological impact of PYY secretion would be significantly altered. Finally we find that the RAPID method combined with MS minimizes exogenous processing and the mass spectrometry gives an efficient way to look for specific or expected peptide processing products. The combination of these approaches assures an accurate assessment of the forms and levels present in tissue and blood.

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Figure 1.

Peptide processing that occurs to produce the possible endogenous forms of PYY. In three enzymatic steps from the prepropeptide a glycine-extended version of PYY is formed. Peptidylglycine α -amidating monooxygenase (PAM) transforms the C-terminus of glycine-extended PYY into an amide. DDP-IV converts PYY(1–36)-NH₂ into PYY(3–36)-NH₂ in a subsequent step in the secretory granules or after the peptide is released into blood.

Figure 2.

Initial purification steps of material extracted from the lower half of rat small intestine (7 g) with isotopically enriched internal standards (IES) present in the extraction buffer. The tissue was extracted with the addition of radiolabeled peptide and IES standards as described in the methods section and loaded onto disposable 10 g C18 cartridges. As shown in A, the PYY-LI (circles) and radioactivity (squares) were eluted by 80% ACN. The 4 mL fractions were pooled starting one fraction before the elution of the radioactivity and stopping one fraction after, diluted 8-fold, and loaded onto a semi-preparative C4 column. As shown in B, PYY-LI was eluted with a gradient of 10% to 90% ACN at a flow rate of 2 mL/min over 180 minutes with fractions collected every 0.5 minutes. Aliquots (10 μ L) were taken for radioimmunoassay and the major PYY-LI peak (circles) was shown to elute at 24.6% ACN.

Figure 3.

In the final purification step, the PYY-LI containing fractions from the C4 column (Figure 2) were pooled, diluted 4×, and loaded onto an analytical phenyl column. The column was eluted with a gradient of 20 to 30% ACN with a flow rate of 1 mL per minute over 100 minutes and fractions were collected every 1 minute. Aliquots (10 μL) were taken for radioimmunoassay and the PYY-LI (circles) eluted at approximately 26% ACN.

Processing compartments for ProPYY

Figure 4.

Aliquots of the PYY-LI fractions from the phenyl step containing PYY(3-36) and PYY(1-36) were pooled (Figure 3), partially dried down and injected into the nLC-ESI-FT-MS instrument. Panel A.) Shows the m/z 796–866 region of the full spectrum (m/z 350–2000) produced as follows: An extracted ion-current chromatogram was generated from the total ion-current chromatogram by filtering for scans which contained the 5+ m/z signal of endogenous PYY(1-36)-NH₂ (849.04 ± 0.1 m/z). The ±0.1 m/z range represents the approximate full width at half height (FWHH) of the 849.04 signal. Because all the forms studied co-elute on C18 with the gradient used the summed scans across the m/z 849.04 containing peak contain the 5+ m/z signals for all the forms. Ratios of peak intensities

from $^{13}\text{C}_{12}$ -PYY(1–36)-NH₂ and $^{13}\text{C}_{12}$ -PYY(3–36)-NH₂ were used to calculate endogenous peptide amounts. No signal was observed for endogenous PYY(3–36)-Gly-OH. Because the amount of PYY(1–36)-Gly-OH was small the amount of this endogenous form was calculated from a separate experiment using the same sample where a narrow m/z 846.50 to 876.51 mass range was scanned (narrow SIM). Panel B.) The 859.8 to 864.5 amu portion of this spectrum is shown with the endogenous PYY(1–36)-Gly-OH and the synthetic $^{13}\text{C}_{12}$ -PYY(1–36)-Gly-OH standard 5+ m/z signals.

Table 1

Table of isotopically enriched standards synthesized for rat PYY tissue extraction.

¹³ C ₁₂ - PYY Standards	Sequence
¹³ C ₁₂ - PYY(1–36)-Gly-OH	YP <u>A</u> K <u>P</u> E <u>A</u> P <u>G</u> E <u>D</u> <u>A</u> S <u>P</u> E <u>E</u> L <u>S</u> R <u>Y</u> <u>Y</u> <u>A</u> S <u>L</u> R <u>H</u> <u>Y</u> <u>L</u> <u>N</u> <u>L</u> <u>V</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> <u>G</u> -OH
¹³ C ₁₂ - PYY(3–36)-Gly-OH	<u>A</u> K <u>P</u> E <u>A</u> P <u>G</u> E <u>D</u> <u>A</u> S <u>P</u> E <u>E</u> L <u>S</u> R <u>Y</u> <u>Y</u> <u>A</u> S <u>L</u> R <u>H</u> <u>Y</u> <u>L</u> <u>N</u> <u>L</u> <u>V</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> <u>G</u> -OH
¹³ C ₁₂ - PYY(1–36)-NH ₂	YP <u>A</u> K <u>P</u> E <u>A</u> P <u>G</u> E <u>D</u> <u>A</u> S <u>P</u> E <u>E</u> L <u>S</u> R <u>Y</u> <u>Y</u> <u>A</u> S <u>L</u> R <u>H</u> <u>Y</u> <u>L</u> <u>N</u> <u>L</u> <u>V</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> -NH ₂
¹³ C ₁₂ - PYY(3–36)-NH ₂	<u>A</u> K <u>P</u> E <u>A</u> P <u>G</u> E <u>D</u> <u>A</u> S <u>P</u> E <u>E</u> L <u>S</u> R <u>Y</u> <u>Y</u> <u>A</u> S <u>L</u> R <u>H</u> <u>Y</u> <u>L</u> <u>N</u> <u>L</u> <u>V</u> <u>T</u> <u>R</u> <u>Q</u> <u>R</u> <u>Y</u> -NH ₂

Table 2

Calculated and observed masses of endogenous and standard rat PYY peptides.

PEPTIDE	ELEMENTAL COMPOSITION	Calculated Monoisotopic molecular weight	Observed Monoisotopic molecular weight	Mass Error $\frac{\text{Obs}-\text{Calc}}{\text{Calc}}$ (ppm)
PYY(1–36)-NH ₂	C ₁₉₀ H ₂₈₈ N ₅₄ O ₅₇	4238.1297	4238.1292	0.13
¹³ C ₁₂ -PYY(1–36)-NH ₂		4250.1700	4250.1696	0.08
PYY(3–36)-NH ₂	C ₁₇₆ H ₂₇₂ N ₅₂ O ₅₄	3978.0136	3978.0169	0.83
¹³ C ₁₂ -PYY(3–36)-NH ₂		3990.0539	3990.0520	0.48
PYY(1–36)-Gly-OH	C ₁₉₂ H ₂₉₀ N ₅₄ O ₅₉	4296.1352	4296.1418	1.52
¹³ C ₁₂ -PYY(1–36)-Gly-OH		4308.1755	4308.1756	0.02
PYY(3–36)-Gly-OH	C ₁₇₈ H ₂₇₄ N ₅₂ O ₅₆	4036.0191	n.d.	n.d.
¹³ C ₁₂ -PYY(3–36)-Gly-OH		4048.0594	4048.0595	0.02

n.d. = not detected

Table 3

Levels of PYY molecular forms in rat and dog intestine

PYY Form	Tissue Level Rat (pmol/g) (<i>n</i> =3)	% Total in rat distal small intestine	Tissue Level Canine (pmol/g) (<i>n</i> =1)	% Total in canine ileum
PYY(1–36)-NH ₂	64.3 ± 4.5	90	161	79
PYY(3–36)-NH ₂	6.1 ± 0.9	9	10	5
PYY(1–36)Gly-OH	0.9 ± 0.1	1	32	16
PYY(3–36)Gly-OH	n.d.	-	n.d.	-

n.d.= not detected