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RAPID HIGH-YIELD PURIFICATION OF NEUROPEPTIDES FROM CANINE INTESTINAL MUSCLE

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SUMMARY

A simplified method for purification of gastrointestinal neuropeptides from relatively small amounts of tissue is described. Sequential adsorption, gel filtration, ion-exchange chromatography, and high-performance liquid chromatography without lyophilization produced good yields of somatostatin, bombesin, and vasoactive intestinal polypeptide immunoreactivity. The method is suitable for simultaneous purification of several small, basic peptides from limited amounts of starting material.

INTRODUCTION

Vasoactive intestinal polypeptide [1], somatostatin [2], and bombesin [3] are polypeptides which were first purified from hog intestine, ovine hypothalamus, and frog skin, respectively. Localization of their immunoreactivities to nerves in brain and gut tissues [4—11] has prompted speculation that they may function as neurotransmitters. Most biological studies to measure effects of these peptides are performed in species other than those from which the peptides were purified, despite evidence for species differences in molecular structure [12—14]. Furthermore, multiple molecular forms of these peptides have been described [14—16]. However, low tissue concentrations and limited tissue availability have discouraged attempts at purification of these peptides from other species. This paper describes a relatively easy and efficient technique for the purification of these peptides, presumably of nervous origin, from the muscle layers of canine small intestines.

EXPERIMENTAL

Equipment

High-performance liquid chromatograph. An Altex Scientific (Berkeley, CA, U.S.A.) Model 312 MP liquid chromatograph equipped with a 210 sample injector and an Altex Hitachi 155-40 detector was employed. All separations were performed on a reversed-phase C-18 Ultrasphere ODS column (5 μm particle size, 250 × 10 mm) also from Altex.

Vacuum centrifuge. A Speed Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.) Model SVC100H was used to concentrate purified peptide solutions.

Reagents and chemicals

Acetone, ammonium acetate, and phosphoric acid were from Mallinckrodt (St. Louis, MO, U.S.A.) HPLC-grade water and acetonitrile were from Baker (Phillipsburg, NJ, U.S.A.). Synthetic bombesin, somatostatin-14, somatostatin-28, and vasoactive intestinal polypeptide were from Peninsula Labs. (San Carlos, CA, U.S.A.). Synthetic gastrin releasing peptide was a gift from Dr. J. Rivier (Salk Institute). Amberlite XAD-2 was from Sigma (St. Louis, MO, U.S.A.). CM-Cellulose was from Whatman (Clifton, NJ, U.S.A.). Sephadex G-50 was from Pharmacia (Piscataway, NJ, U.S.A.).

Methods

Radioimmunoassay. Bombesin-like immunoreactivity (BLI), somatostatin-like immunoreactivity (SLI), and vasoactive intestinal polypeptide-like immunoreactivity (VLI) were measured by radioimmunoassay as previously described [17] using antibodies 1078 which is specific for the carboxyl terminus of bombesin, 7812 [18] which is specific for the ring-portion of somatostatin, and 7913 [14] which is specific for the carboxyl terminus of vasoactive intestinal polypeptide.

Tissue extraction. Two dogs weighing 18 and 20 kg were killed by injection with Repose (6 ml i.v.). The entire small intestine was removed and immersed in boiling water (5 ml/g) within 15 min of sacrifice. After boiling for 5 min the small intestine was opened and the mucosa layer was removed from the muscle by scraping. The muscle was blended in 4.5% acetic acid (2 ml/g) for 3 min; then acetone was added (7 ml/g) and the mixture stirred for 6 h at 4° C. The tissue was centrifuged for 30 min at 3000 *g* at 6° C. Acetone was removed from the supernatant by rotary evaporation. The remaining supernatant was centrifuged for 1 h at 3000 *g* at 6° C.

Amberlite XAD-2 chromatography. The concentrated extract was loaded directly onto an Amberlite XAD-2 column (30 × 5 cm) that had been washed with acetone, methanol and equilibrated with water. After loading, the column was rinsed with water until the absorbance at 280 nm (A_{280}) was less than 0.05. The column was then eluted with 100% methanol (1 l) until eluate had an A_{280} of less than 0.01. Glacial acetic acid was added (2 ml) to the pooled methanol eluate which was then concentrated to 65 ml by rotary evaporation and centrifuged for 1 h at 3000 *g* at 6° C.

Sephadex G-50 chromatography. The supernatant was loaded onto a Sepha-

dex G-50 SF column (5 × 140 cm) and eluted with 1% acetic acid at a flow-rate of 80 ml/h. Eluted fractions (10 ml) were assayed for BLI, SLI, and VLI. The elution profile of this column is shown in Fig. 1.

CM-Cellulose chromatography. Fraction I, containing VLI, and fraction II, containing SLI-I and BLI-I (Fig. 1) were separately chromatographed on a CM-cellulose column equilibrated in 0.1 *M* ammonium acetate, pH 5.0. The columns were rinsed with 200 ml 0.1 *M* ammonium acetate, pH 5.0, and eluted with a 300-ml linear gradient of ammonium acetate (0.1—1 *M*, pH 5.0) at a flow-rate of approximately 20 ml/h. Fractions (5 ml) were collected, and assayed for the immunoreactive peptides.

Gradient HPLC. CM-Cellulose-purified BLI-I, SLI-I, and VLI were loaded onto a C-18 reversed-phase HPLC column equilibrated in 0.25 *M* triethylaminophosphate, pH 3 (TEAP). After loading 75—100 ml samples, the column was rinsed with TEAP and then eluted with a linear gradient of acetonitrile from 0% to 30% over 60 min, then at 30% for another 30 min at a flow-rate of 2 ml/min. Fractions of 2 ml were collected and assayed for immunoreactive peptides. Peptides eluted 1—12 min after reaching 30% acetonitrile.

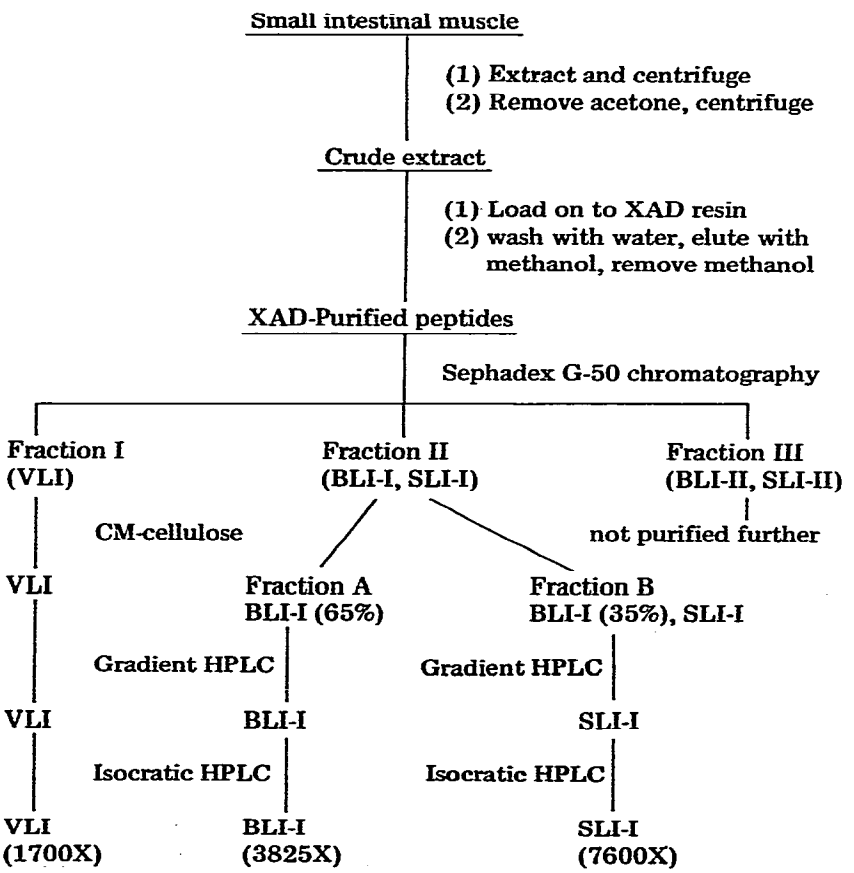


Fig. 1. Flow diagram of the purification procedure.

Isocratic HPLC. Gradient-purified BLI-I, SLI-I, and VLI were each pooled and concentrated to 3 ml in a Speed-Vac Concentrator. These concentrates were chromatographed isocratically on a C-18 reversed-phase HPLC column with 74% TEAP and 26% acetonitrile at a flow-rate of 1 ml/min. Fractions of 1 ml were collected and assayed for immunoreactive peptides.

RESULTS

A flow diagram of the purification procedure is depicted in Fig. 1. As shown in Table I, the acid—acetone extracts of intestinal muscle from two dogs (462 g) yielded 101 nmol of VLI, 16.2 nmol of BLI, and 7.6 nmol of

TABLE I

RECOVERY OF BLI, SLI AND VLI FROM 462 g CANINE SMALL INTESTINAL MUSCLE

Step	Immunoreactivity (nmol)		Step recovery (%)	Cumulative recovery (%)	nmol/A_{280}	Purification ratios (X)
	Total BLI	BLI-I				
Extract	16.2		100	100	0.004	1
XAD	14.7		91	91	0.035	8.8
G-50	13.2	9.9*	88	81	0.31	77.5
CM-Cellulose		9.4	95	77	2.1	525
Gradient HPLC		5.8	62	36	7.7	1925
Isocratic HPLC		4.1	80	28	15.3	3825
	Total SLI	SLI-I				
Extract	7.6		100	100	0.002	1
XAD	6.9		90	90	0.016	8.0
G-50	6.3	3.8*	92	83	0.15	75
CM-Cellulose		3.7	97	80	0.74	370
Gradient HPLC		3.5	95	76	2.3	1150
Isocratic HPLC		3.1	89	67	15.2	7600
	VLI					
Extract	101		100	100	0.025	1
XAD	66		65	65	0.16	6.4
G-50	60		91	59	4.0	160
CM-Cellulose	45		75	44	9.3	372
Gradient HPLC	40		90	40	33.3	1333
Isocratic HPLC	31		77	31	30	1700

*Cumulative recovery beyond this step was calculated for the larger components, BLI-I and SLI-I, separated from smaller forms by gel filtration.

SLI. Amberlite XAD-2 chromatography resulted in a 6—8 fold purification of the peptides with minimal losses except for VLI (35% loss). On Sephadex G-50 chromatography (Fig. 2), VLI eluted as a single peak (Fraction I) which was separated from the bulk of A_{280} and from the SLI and BLI peaks. Both

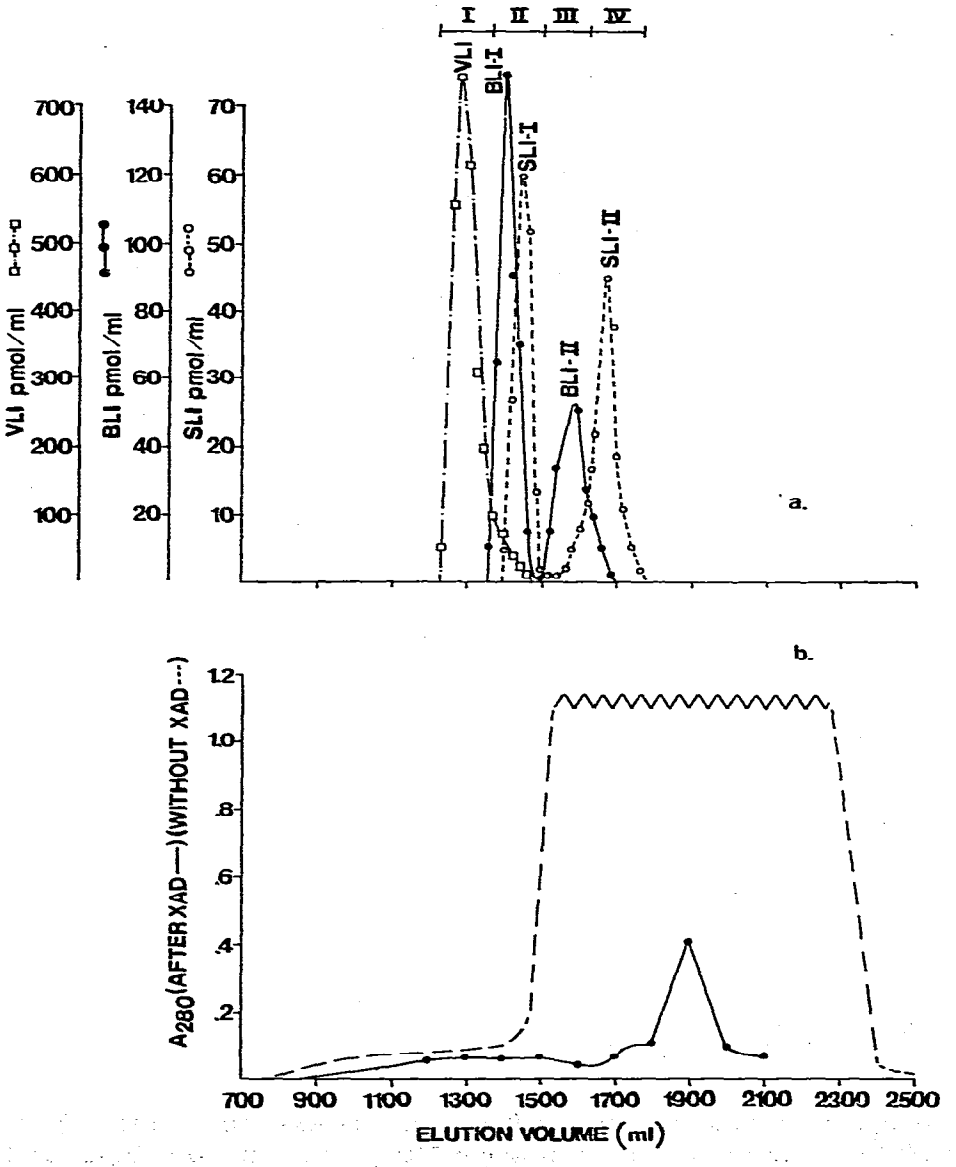


Fig. 2. Chromatography of Amberlite XAD-2 concentrate on a Sephadex G-50 column (140 × 5 cm) eluted with 1% acetic acid. (a) The immunoreactivity profile for VLI (· — · —), BLI (———) and SLI (— — —). (b) The absorption profile at 280 nm for the same XAD-2 concentrate (———), and for an acid—acetone extract from another preparation of intestine that had not been applied to XAD-2 but lyophilized to dryness, dissolved in 1% acetic acid, clarified by centrifugation and then chromatographed on the same column (— — —).

BLI and SLI eluted as two peaks, the larger forms (SLI-I and BLI-I) co-eluted in Fraction II, while the smaller forms (SLI-II and BLI-II) were relatively well separated. The peptides were purified 9—25 fold by Sephadex G-50 chromatography and the recoveries were nearly quantitative.

Fraction I from the Sephadex G-50 chromatography, containing 90% of the VLI, was sequentially purified by CM-cellulose chromatography, gradient HPLC, and isocratic HPLC (see Tables I and II). VLI eluted as a single peak in all these steps with good recovery. However, a contaminant, possibly from the buffer system, co-eluted with the VLI during the isocratic HPLC step, and there was no apparent purification at this step. The final purification of VLI was 1200-fold with a 31% net yield.

BLI and SLI-I, which co-eluted during Sephadex G-50 chromatography, were partially resolved by CM-cellulose chromatography (see Table II). One fraction contained 65% of the BLI-I, and the other contained 35% of the BLI-I and nearly all of the SLI-I. The low recovery of BLI-I following gradient HPLC can be explained because of only 65% of the material recovered from CM-cellulose chromatography was used. Otherwise, the yields for the two HPLC steps were above 80%. Purification of BLI-I was 3825-fold with a net yield of 28% and SLI-I was purified 7600-fold with a net yield of 67%. Unique absorbance peaks were associated with the immunoreactive profile for BLI-I at 13 min (Fig. 3) and for SLI-I at 19 min (Fig. 4) when isocratic elution was done at 26% acetonitrile. The ratios of immunoreactivities to A_{280} suggested that the peptides were nearly pure, assuming the same extinction coefficient for each peptide as their known counterparts.

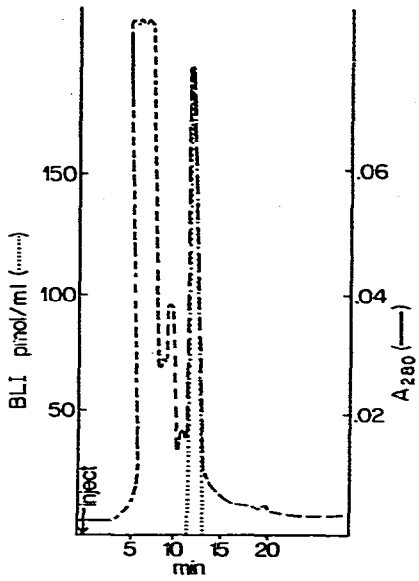
TABLE II

ELUTION POSITION OF THE IMMUNOREACTIVE PEPTIDES DURING PURIFICATION

	VLI	BLI-I	SLI-I
G-50 peak (%)*	52	59	63
(Range)	(44—66)	(55—65)	(57—67)
CM peak**	0.45	0.33	0.40
(Range)	(0.39—0.52)	(0.15—0.43)	(0.26—0.53)
Gradient HPLC			
(min after 30%)	1	6	12
Isocratic HPLC (min)	9	13	19

*Percentage from protein to salt peak.
**Concentration of ammonium acetate (*M*).

It was found that immunoreactive VIP is homogeneous and similar in gel permeation elution characteristics to porcine VIP while both bombesin and somatostatin immunoreactivities eluted in two major forms (Fig. 2). The larger forms of BLI eluted in a similar position to porcine gastrin releasing peptide while the larger form of SLI eluted in a similar position to porcine somatostatin 28 (Table III). The smaller forms eluted in similar positions testing bombesin (BLI-II) and to somatostatin 14 (SLI-II).



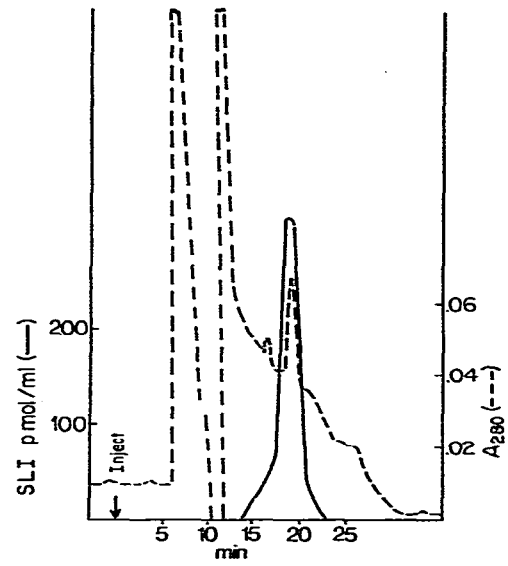


Fig. 3. Isocratic HPLC elution profile of the gradient-purified BLI. The dotted line shows the immunoreactive profile, and the dashed line the absorbance at 280 nm. Details of the chromatography are in the Methods section.

Fig. 4. The isocratic HPLC elution profile of the gradient purified SLI. The dotted line shows the immunoreactive, and the dashed line the absorbance at 280 nm. Details of the chromatography are in the Methods section.

TABLE III

ELUTION POSITIONS OF EXTRACTED PEPTIDES AND STANDARD PEPTIDES ON SEPHADEX G-50 COLUMNS

Peptide	Elution (%)*
Porcine VIP	44
Canine VLI	44
Porcine gastrin releasing peptide	53
Canine BLI-I	56
Porcine somatostatin 28	50
Canine SLI-I	50
Frog bombesin 14	76
Canine BLI-II	83
Porcine somatostatin-14	86
Canine SLI-II	86

*Percentage from protein to salt peak on an analytical G-50 (SF) column (100 × 1 cm) eluted with 3% acetic acid.

DISCUSSION

We have demonstrated that substantial quantities of three neuropeptides can be purified from limited amounts of tissue. Canine intestinal muscle VLI, BLI, and SLI have been purified several thousand times with final recoveries ranging from 28—67%. Indeed, all three peptides would have been isolated with yields of better than 50% if only one peptide had been purified at a time. Such yields suggest that our protocol may be applied to purify peptides from material that is not readily available such as fresh human tissues or unusual hormone-secreting tumors.

Rigorous maintenance of acidic conditions was required to achieve optimal recoveries of these basic peptides. Aside from inevitable losses caused by multiple isolation from single extracts, losses greater than 10% occurred only at steps that required concentration of peptide solutions. Thus, Amberlite XAD-2 chromatography was a particularly useful first step in purification since it resulted in a 6—8 fold purification and concentrated the solution approximately 4-fold without the losses that occur during lyophilization or rotary evaporation.

The HPLC steps resulted in a substantial purification of peptides with minimal losses. Apparent contamination of VLI in the final isocratic HPLC step was of some concern. To avoid further contamination, new HPLC systems for purification of VIP are currently under investigation. This relatively simple, rapid, and efficient protocol may be useful for future purifications of numerous polypeptides from a variety of tissues available only in limited quantities. This should prove to be especially useful in the characterization of human peptides.

The results furthermore indicate that immunoreactive VIP in canine muscle is homogeneous while immunoreactive somatostatin and bombesin are heterogeneous, with about 70% respectively present as large forms. This purification scheme is suitable for purification of both large and small forms of heterogeneous basic neuropeptides.

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REFERENCES

1 S.I. Said and V. Mutt, Eur. J. Biochem., 28 (1972) 199.
2 P. Brazeau, W. Vale, R. Burgas, N. Ling, M. Bretcher, J. Rivier and R. Guillemin, Science, 179 (1973) 17.
3 A. Anastasi, V. Erspamer and M. Bucci, Experientia, 27 (1971) 166.
4 M.G. Bryant, J.M. Polak, I. Modlin, S.R. Bloom and R.H. Albuquerque, Lancet, i (1976) 991.
5 H. Fuxe, T. Hokfelt, S.I. Said and V. Mutt, Neurosci. Lett., 5 (1977) 241.
6 L.-I. Larson, J. Fahrenkrug, O. Schaffalitzky de Muckadell, F. Sundler, R. Hakanson and J.F. Rehfeld, Proc. Nat. Acad. Sci. U.S., 73 (1976) 3197.

7 M.I. Grossman, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 2343.
8 B.L. Punitone, M. Sheppard, B. Shapiro, S. Kronhein, A. Hudson, S. Hendricks and K. Waligora, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 2330.
9 M. Brown and W. Vale, Trends in Neurosci., 2 (1979) 95.
10 J.H. Walsh, H.C. Wong and G.J. Dockray, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 2315.
11 T.W. Moody and C.B. Pert, Biochem. Biophys. Res. Commun., 90 (1979) 7.
12 R. Dimaline and G.J. Dockray, Life Sci., 25 (1979) 1893.
13 R.H. Goodman, J.W. Jacobs, W.W. Chin, P.K. Land, P.C. Doe and J.F. Habener, Proc. Nat. Acad. Sci. U.S., 77 (1980) 5869.
14 R. Dimaline and G.J. Dockray, Gastroenterology, 75 (1978) 387.
15 E.S. Zyznar, J.M. Conlon, V. Schusdziarra and R.A. Unger, Endocrinology, 105 (1978) 1426.
16 J.H. Walsh, J.R. Reeve, S.R. Vigna, P. Chew, H.C. Wong and G.J. Dockray, Scand. J. Gastroent., 13 (S49) (1978) 191.
17 J.H. Wals and H.C. Wong, in B.M. Jaffe and H.R. Behrmann (Editors), Methods of Hormone Radioimmunoassay, Academic Press, New York, 1979, pp. 581—593.
18 T. Yamada, D. Marshak, S. Basinger, J. Walsh, J. Morley and W. Stell, Proc. Nat. Acad. Sci. U.S., 77 (1980) 1691.