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PURIFICATION OF LARGE NEUROMEDIN N (NMN) FROM CANINE INTESTINE AND ITS IDENTIFICATION AS NMN-125**

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SUMMARY: A large molecular form of neuromedin N (NMN), a neurotensin (NT)-related peptide, was purified to homogeneity from canine ileal mucosa. The amino acid sequence of its N-terminal 20 residues was found to be SDSEEEMKALEADLLTNMHT, which is identical to residues 24-43 of the cDNA-predicted NT/NMN precursor. Prior work had indicated that the NMN moiety was located at the C-terminus of large NMN. Having now defined both the N-terminus and C-terminus of large NMN, we note that this molecule must be comprised of 125 amino acids (including residues 24-148 of prepro NT/NMN) and we suggest that it be named NMN-125. This information also defines the signal peptide cleavage site as the cysteine^{23}-serine^{24} bond within the precursor.

The amino acid composition of the isolated peptide and its molar extinction coefficient for absorbance at 280 nm were similar to those for the 24-148 segment of prepro NT/NMN. However, the empirically determined molecular weight (17 kDa) and the isoelectric point (pI = 5.4) were slightly higher than those predicted solely from the peptide's amino acid content (14.43 kDa and pI = 4.65, respectively). In total, these results indicate that the major NMN-related peptide found in canine ileum is 125 residues in length, extending from the putative signal peptide to the C-terminus of NMN, and that it might contain non-amino acid substituents.

Neuromedin N (NMN) , a hexapeptide in the neurotensin (NT) family (1), exists primarily (>75%) as a large molecular form of ~17 kDa in the canine small intestine (2). Although the NMN-moiety has been shown to be present at the immediate C-terminus of large NMN (2), the entire molecule has never been isolated and its precise size has not been established. The present study describes a procedure for its isolation from canine ileum and presents the sequence of its first 20 N-terminal amino acid residues, which are identical to residues 24 to 43 of prepro-NT/NMN as predicted from cDNA work (3).

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The abbreviations used are: NMN, neuromedin N; NT, neurotensin; iNMN, immunoreactive NMN; RIA, radioimmunoassay; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Radioimmunoassay: RIA was performed as previously described for NMN using antiserum TG-B (5). Assay sensitivity was 20 fmol/tube and the dose-response for large NMN was parallel to that for synthetic NMN over a 10-fold range.

Purification Procedure: Canine ileal mucosa (100 g) was dissected from adult dogs as previously described (2) and immediately extracted with 10 volumes (vol/wt) of ice-cold 0.1N HCl containing 10 µg/ml pepstatin A. The extract was placed in boiling water for 15 minutes and stored at -20°C. A day later, it was thawed and centrifuged at 10,000 xg for 15 minutes. The supernatant was then subjected to gel chromatography on an 18L column of Sephadex G-75 in 5% acetic acid, collecting fractions of 0.5 L. The active material, eluting at 12-14 L, was concentrated to 300 ml by rotary evaporation and subjected as 2 batches to reverse-phase HPLC using Water's equipment (Millipore Corp., Milford, MA.). The Delta Pak-C18-100A column (19 x 300 mm) was developed at 6 ml/min with a 10 min linear gradient from 0.1% trifluoroacetic acid to 15% CH_3CN, followed by a 120 min gradient to 75% CH_3CN. The active material eluting at ~60% CH_3CN, was concentrated and applied to a Delta Pak C4-300A column (7.8 x 300 mm) equilibrated with 0.1% trifluoroacetic acid. The column was eluted at 2.5 ml/min using a 5 min gradient to 15% CH_3CN, followed by a 120 min gradient to 75% CH_3CN. The active material, eluting at ~44% CH_3CN, was subjected to ion exchange chromatography on a column (7.5 x 75 mm) of Biogel TSK-SP-5-PW equilibrated at 1.5 ml/min with 4% acetic acid (pH 2.9), 25% CH_3CN. The eluting buffer (B) was 2.5 mM tris-acetate (pH 4.0), 12% guanidine-HCl, 25% CH_3CN. The program was a 20 min gradient to 50% B, a 5 min hold and a 15 min gradient to 100% B. The active material, which eluted at 75% B, was re-run on the Delta Pak C4-300A as described above and then re-run on the TSK-SP-5-PW using a slightly different program. This involved a 20 min gradient to 50% B, a 5 min hold, a 4 min gradient to 63% B, a 4 min hold and an 11 min gradient to 100% B. As a final step, the active material was re-run on the Delta Pak C4, as above. During the final 3 HPLC steps, fractions were collected at 0.5 min intervals using polyethylene tubes.

Protein Sequencing: Sequencing was performed using an Applied Biosystems 477A sequencer and 420 amino acid analyzer at the Microchemistry Facility, Harvard University Biological Laboratory, Cambridge, MA.

Isoelectric Focusing: A Biorad ROTOFOR System was used with a pH gradient from 3-10 generated in 1% pharmalyte. Large NMN (0.3 nmol, post HPLC) was dissolved in 1 ml of 6 M urea and 0.1% triton X-100, then diluted to 50 ml with water, loaded and focused for 5 hrs at 4°C using 12 watts.

SDS-gel Electrophoresis: This was performed as previously described using a 20% polyacrylamide slab gel (2). The proteins were electrophoretically transferred to nitrocellulose paper using a Hoefer apparatus with 1 amp for 4 hrs. at 4°C. Incubation with primary antibody was done at 1:100 dilution in 1% milk for 48 hrs. Incubation with biotinylated secondary antibody was performed as recommended by Vectastain elite kit (Vector Labs, Burlingame, CA). The strips were finally stained using ABC reagents and 0.1% diaminobenzidine with 0.01% H_2O_2.

Computer-assisted Calculations: The values for the molecular weight, pI, mole % amino acid composition and the secondary structures predicted by the Chou and Fasman algorithm for prepro NT/NMN (24-148) were obtained using the IntelliGenetics computer program, Polypeptide Analysis System.

RESULTS

Purification: When subjected to gel chromatography on Sephadex G-75, ~90% of the NMN-related activity present in an acid extract of canine ileum eluted as a large-molecular form (Fig. 1). This substance was purified to

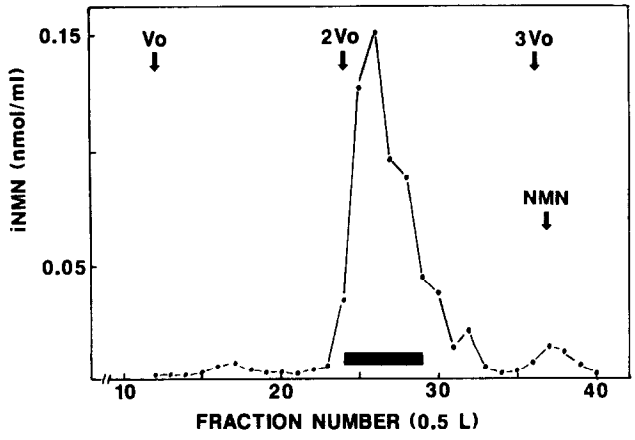


Figure 1. Profile of iNMN activity during gel chromatography of an HCl extract of canine intestine on Sephadex G-75. See text for details. The active region, which was pooled for further purification, is indicated by a black box.

homogeneity using the scheme described in Methods and summarized in Table I, by monitoring iNMN using the RIA. A 50,000-fold purification was achieved with a yield of ~3%. The peptide was judged pure based upon (a) its maximal specific activity (Table I); (b) coincident peaks of absorbance and immunoreactivity in the final step; and (c) the finding of a single N-terminal amino acid (serine) by Edman analysis.

Amino Acid Composition: The amino acid composition determined for isolated large NMN was in good agreement with that predicted for the (24-148) segment of prepro-NT/NMN (Table II).

Sequence: Edman degradation performed on two independently isolated preparations unambiguously established the sequence of the N-terminal 20 residues of the isolated peptide which were identical to residues 24-43 of the cDNA predicted sequence of prepro-NT/NMN (Table III and Fig. 2). Since the NMN moiety was known to be located at the C-terminus of this molecule, it was concluded that large NMN was 125 residues in length and the name "NMN-125" was suggested.

TABLE I

Purification procedure for isolation of large NMN from 100g of canine ileal mucosa

Purification Step	iNMN (nmol)	Yield (%)	Specific Activity (nmol/ml x absorbance)
Crude extract	83	100	0.003
G-75 - Sephadex	71	86	0.04
C18 - reverse-phase	23	28	0.4
C4 - reverse-phase	12	14	5.5
SP - ion exchange	5.2	6	95.
C4 - reverse-phase	2.2	3	181.

Specific activity is given as nmol iNMN/ml x absorbance at 280 nm.

TABLE II

Comparison of the Amino Acid Composition for Isolated Large NMN and that predicted for Prepro NT/NMN (24-143)

AMINO ACID	MOLE %		RESIDUE #
	EMPIRICAL	THEORETICAL**	
Asx	9.6	10.7	13
Glx	16.4	18.2	22
Ser	9.5	9.1	11
Gly	4.2	2.1	3
His	1.8	1.7	2
Arg	5.1	4.1	5
Thr	5.9	5.8	7
Ala	7.5	6.6	8
Pro	2.8	1.7	2
Tyr	1.4*	1.7	2
Val	4.8	4.1	5
Met	1.5	3.3	4
Cys	--	1.6	2
Ile	6.2	6.6	8
Leu	11.8	11.6	14
Phe	4.3	4.1	5
Lys	8.5	8.3	10
Trp		--	2
Total			125

Pure large NMN (200 pmol) was hydrolyzed in 6N HCl made 1% phenol for 24 hrs at 110°C and the amino acids were quantitated.

* Tyrosine value corrected for 70% yield during hydrolysis.

** Tryptophan was excluded since it is destroyed during acid hydrolysis.

TABLE III

Amino acid sequence analysis of large NMN. Edman degradation was performed on two independently-isolated preparations of peptide and the yield of the major PTH-amino acid obtained at each cycle is given

CYCLE	RESIDUE	YIELD (pmol)*	
		#1	#2
1	Ser	128	---
2	Asp	108	1.9
3	Ser	91	2.1
4	Glu	97	1.3
5	Glu	87	1.3
6	Glu	75	1.3
7	Met	66	1.1
8	Lys	58	0.9
9	Ala	65	1.5
10	Leu	60	1.2
11	Glu	36	0.4
12	Ala	48	1.0
13	Asp	42	0.4
14	Leu	40	1.0
15	Leu	50	0.9
16	Thr	42	0.4
17	Asn	26	0.7
18	Met	20	0.5
19	His	8	
20	Thr	8	

*#1 and #2 were different preparations of peptide. #1 (166 pmol) was sequenced by gas-phase. #2 (3 pmol) was coupled via C-terminus and sequenced by solid-phase.

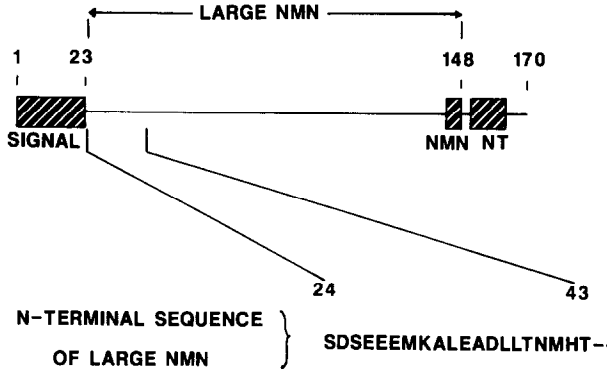
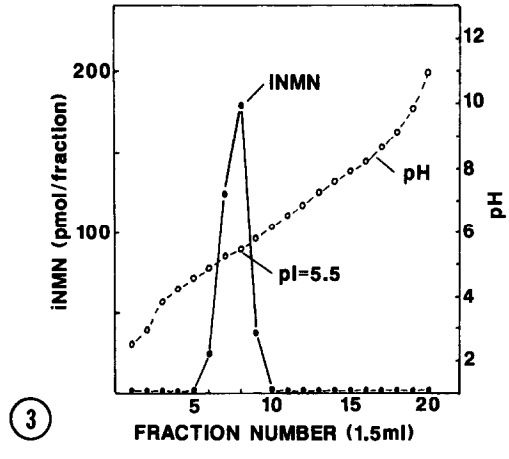


Figure 2. Diagram showing relationship between large NT/NMN precursor (upper) based upon the amino acid sequence determined for the N-terminal 20 residues of large NMN (lower). Since this sequence was identical to that for residues 24-43 of the precursor, large NMN must extend from residues 24 to 148.

Isoelectric Point: Two preparations of highly purified NMN-125 gave isoelectric points of 5.5 (Fig. 3) and 5.3 (not shown). The pI predicted from the amino acid content of prepro NT/NMN (24-148) was 4.65.

Molecular Weight: The molecular weight determined for highly-purified NMN-125 using SDS-gel electrophoresis and Western blot analysis was ~17 kDa (Fig. 4). This was slightly larger than that predicted from the amino acid content of the cDNA-predicted sequence (14.43 kDa).



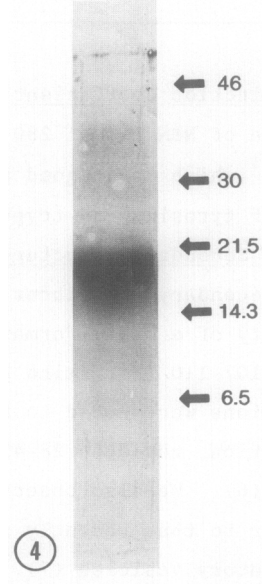


Figure 3. Profile of iNMN activity during isoelectric focusing of partially-purified large NMN (~ 300 pmol iNMN post HPLC).

Figure 4. Western blot and antibody-peroxidase detection of large NMN. Large NMN (~ 200 pmol iNMN post HPLC) was subjected to SDS-PAGE in a 20% slab gel and after electrophoretic transfer to nitrocellulose paper, the blot was reacted with antiserum TG-B (1:100) and immune complexes were visualized. The major band displayed a molecular mass of ~17kDa.

Table IV

Secondary Structures Predicted For NMN-125
(Amino Acid Sequence Given at Bottom of Table)

Begin	End	Type	<Pa>	<Pb>	<Pt>
2	16	alpha	1.249	.790	
13	17	beta	.986	1.044	
19	22	turn	.940	.887	1.087
21	26	alpha	1.060	.902	
28	31	turn	.920	1.143	1.080
29	36	alpha	1.060	1.056	
33	39	beta	1.019	1.231	
41	46	beta	.902	1.175	
45	52	alpha	1.129	.850	
50	66	alpha	1.173	.855	
60	64	beta	1.016	1.190	
66	70	beta	1.032	1.050	
69	81	alpha	1.140	1.015	
77	85	beta	1.123	1.216	
87	106	alpha	1.125	1.039	
89	92	turn	1.043	.845	.998
105	108	turn	.917	.752	1.310
107	110	turn	.852	.730	1.398
109	121	alpha	1.158	.872	
109	112	turn	1.088	.635	1.192
121	125	beta	.926	1.304	

<Px> = average propensity for x = a(α-helix), b(B-sheet), or t(turn).

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        10        20        30        40        50        60        70
SDSEEEMKALEADLLTNMHTSKISKASVSSWKMTLLNVCSFVNNLNSQAEETGEFREEELITRRKFPTAL

                80        90       100       110       120
DGFSLEAMLTIYQLQKICHSRAFQQWELIQEDVLDAGNDKNEKEEVIKRKIPYIL
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Molar Extinction Coefficient: The molar extinction coefficient for the absorbance of NMN-125 at 280 nm and pH 2 was estimated to be 10 x $10^3 M^{-1} cm^{-1}$ which is in good agreement with that predicted from the content of tyrosine and tryptophan (2 each/mol; ϵ = 12.5 x $10^3 M^{-1} cm^{-1}$).

Predicted Secondary Structure: Using the algorithm by Chou and Fasman (5) to predict secondary structures for NMN-125, we found that there was a high probability of α-helix formation for most of the peptide, with a turn at residues 107-110 (For Amino Acid Sequence and Probabilities, see Table IV). Three regions were noted to be highly amphipathic in the helical configuration, residues 28-45, 69-84 and 92-106, by performing helical wheel analyses (6). We also observed the presence of a motif, residues 3-15, which is similar to that recently suggested to promote the sorting of prohormones into secretory vesicles (7).

DISCUSSION

The work presented here establishes the sequence of the first 20 N-terminal amino acids of large NMN. Coupled with the earlier finding that the NMN-moiety is located at its C-terminus, this information permits one to

deduce that large NMN is comprised of 125 amino acids and that it extends from residue 24 to residue 148 in the cDNA-predicted structure of canine prepro-NT/NMN (Fig. 2). This information also defines the signal peptide cleavage site as the cysteine^{23}-serine^{24} bond, rather than the serine^{24}-aspartic acid^{25} bond originally suggested by Dobner et al (4).

NMN-125 was purified ~50,000-fold using chromatographic steps which were fairly standard for basic peptides (Table I). It was, however, important to perform all procedures under acidic conditions since the peptide tended to aggregate and precipitate at pH's above 4. An ion exchange step was difficult to develop due to the unusual behavior of the molecule, which was not retrieved from carboxymethyl-or sulfopropyl-cation exchangers even with 2M NH_4OH. The finding that a gradient in guanidine-HCl eluted the peptide in good yield from the Biogel TSK-SP ion exchanger, sharply resolving it from basic contaminants, was key to the success of the purification. The homogeneity of the final preparation was attested to by (a) its sharp migration during HPLC, isoelectric focusing (Fig. 3) and SDS-PAGE (Fig. 4); (b) the fact that its amino acid composition (Table II) was similar to that predicted for prepro NT/NMN (24-148); and (c) the fact that a single sequence was obtained for the first 20 N-terminal residues which were identical to those in the predicted peptide (Table III and Fig. 2).

The physico-chemical characteristics of the highly-purified molecule indicated that NMN-125 displayed some anomolous behavior which might be due to additional side-chain substituents or might simply be attributed to its unusual charge distribution and amphipathic nature. Thus, the molecular weight measured by SDS-gel electrophoresis (17 kDa) was somewhat higher than that for the sum of its 125 amino acid residues (14.43 kDa) and the empircally determined pI (5.4) was slightly higher than the theoretical value (4.7). The finding that the absorbance of the isolated peptide at 280 nm gave an estimated extinction coefficient which was consistent with the expected content of tyrosine and tryptophan argues that, if additional substituents are present, they are likely to be non-aromatic.

Predictions concerning the secondary structure of NMN-125 (Table III) suggested that the molecule might present three hydrophobic surfaces along its α-helical configuration, each consisting of 7-8 aligned, non-polar residues. The turn predicted to occur at residues 107-110 would project the NMN-moiety (residues 120-125) away from the main axis, possibly serving to permit interactions with processing enzyme(s) and/or receptor(s). The packaging of this molecule within secretory vesicles might possibly be directed by the prohormone-sorting motif (7) which we found to occupy residues 3-15.

In total, the information presented here indicates that the major storage form of NMN-like activity in canine intestine is a 125 residue peptide

extending from the end of the putative signal sequence in the precursor to the end of the NMN-moiety. The properties of the isolated peptide are not completely consistent with those predicted from its amino acid content and allow for the possible presence of additional substituents of a basic nature. Predictions concerning the secondary structure indicate primarily an α-helical configuration with three highly amphipathic regions and a major turn near the C-terminus which projects the NMN-moiety away from the main axis of the molecule.

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