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ION-EXCHANGE, GEL-FILTRATION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ISOLATION OF NEUROTENSIN-DEGRADING ENZYMES FROM RAT BRAIN

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SUMMARY

Brain and pituitary peptidases involved in the metabolism of neurotensin were identified by ion-exchange (TSK-545 DEAE) and gel-filtration (TSK-G4000 SW) high-performance liquid chromatography (HPLC). Reversed-phase HPLC on radial compression cartridges was used in a rapid assay of neurotensin-hydrolysing activity in column fractions and at the same time the bond specificity of the activity was determined. Semipreparative isolation of the major enzyme which cleaves neurotensin at Arg^8–Arg^9 was carried out and some of its characteristics were determined.

INTRODUCTION

Neurotensin (NT, see Fig. 1), is one of the growing number of neuropeptides thought to act as neurotransmitters or modulators of synaptic events[1]. The mechanisms for controlling the concentration of neuropeptides at the nerve terminal and for terminating their actions are currently unknown but seem certain to involve peptidases[2]. Isolated nerve endings (synaptosomes) contain enzymes which rapidly degrade NT and it has been shown by reversed-phase high-performance liquid chromatography (HPLC) and amino acid analysis of the degradation products that the major primary cleavage point is the Arg^8–Arg^9 bond[3]. In addition, the Pro^{10}–Tyr^{11}, Pro^7–Arg^8, Ile^{12}–Leu^{13} and Tyr^{11}–Ile^{12} bonds are also hydrolysed by synaptosomal enzymes. Further progress in identifying which peptidases are of physiological significance depends upon the isolation and characterization of the enzymes involved. Although endopeptidases have been identified in brain which hydrolyse NT[4–6], their sites of cleavage do not correspond to the major cleavage point (Arg^8–Arg^9) of synaptosomal peptidase activity.

The introduction of stationary phases for high-performance ion-exchange

pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH

1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 1. Structure of neurotensin.

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chromatography[7], that are based on the rigid, hydrophilic TSK-GEL type SW column material[8] offers a rapid and efficient means for the analytical and semi-preparative separation of enzymes. We report here on the use of high-performance ion-exchange (TSK IEX-545DEAE) and gel-filtration (TSK-G4000SW) chromatography to separate NT-degrading enzymes from rat brain and pituitary. The specificity of the peptidase activity in column fractions was determined as follows. Neurotensin was incubated with column fractions and the degradation products rapidly separated and identified by reversed-phase HPLC on μBondapak C_{18} radially compressed cartridges (Radial-Pak with Z-module; Waters Assoc.). Using these methods, three NT-degrading peptidases were identified in rat brain and pituitary extracts. The major enzyme which cleaves the peptide at the Arg^8–Arg^9 bond (Arg–Arg cleaving enzyme, AACE), was further purified by ion-exchange and gel-filtration HPLC, and some of its characteristics were determined. Similar techniques should be applicable to the study of peptidases involved in the metabolism of other neuropeptides.

EXPERIMENTAL

Apparatus

Tissue extracts were chromatographed on TSK-545DEAE (15 × 0.75 cm) and TSK G4000SW (60 × 0.75 cm) columns (both from LKB Instruments, Croydon, Surrey, U.K.) using Waters HPLC equipment (two Model 6000A pumps, U6K injector, Model 660 gradient former) coupled to a Uvicord-S detector (277 nm) and Ultrorac fraction collector (LKB). A guard column (7.5 × 0.75 cm; TSK-GSWP; LKB) was used in both cases.

NT degradation products were separated by reversed-phase HPLC on a Radial-Pak μBondapak C_{18} cartridge fitted to a Z-module radial compression unit (Waters Assoc., Northwich, U.K.). The apparatus consisted of two Model 6000A pumps, a 710B-WISP auto sampler, 730 data module, 720 system controller and Model 450 UV detector (210 nm).

Chemicals

Acetonitrile (HPLC-grade S) was obtained from Rathburn Chemicals, Walkerburn, U.K., neurotensin from Universal Biologicals, Cambridge, U.K., trifluoroacetic acid (TFA), acetic acid and Tris from BDH, Poole, U.K.

Tissue extraction

Three rat brains (3 g total) were homogenized using a glass–PTFE homogenizer in 30 ml of 10 m*M* Tris–acetate buffer (pH 7.2), and the homogenate was centrifuged at 50,000 *g* for 30 min at 4°C. The supernatant was dialysed against 10 m*M* Tris–acetate buffer (pH 7.2), and the non-diffusible material was used immediately for separation studies. Three rat pituitaries were extracted in the same way using 0.5 ml of buffer but without dialysis.

For semi-preparative separations, the brain extract (30 ml) was passed through an open DEAE-cellulose column (5 × 2 cm, Whatman DE52). After washing the column with 20 ml of 0.1 *M* Tris–acetic acid, pH 7.2, the peptidase activity was eluted with 0.35 *M* Tris–acetic acid, pH 7.2 (20 ml). The eluate was dialysed against 0.01% mercaptoethanol and freeze-dried.

Ion-exchange HPLC

Ion-exchange chromatography of proteins on DEAE-modified supports normally employs a sodium chloride gradient at fixed pH to elute the components from the column selectively. We wished to avoid the use of sodium chloride gradients for two reasons: (a) high concentrations of halide ions can damage stainless-steel surfaces and (b) an endopeptidase which degrades NT is known to be sensitive to high sodium ion concentrations[7]. In initial experiments we found that the NT-degrading activity of brain extracts was considerably less stable in high sodium chloride concentrations (0.1–0.5 *M*) than in the same concentrations of Tris–acetic acid (pH 7.2). Therefore gradients of increasing Tris–acetic acid molarity were used in this study.

Brain extract (2 ml) or pituitary extract (0.5 ml) was applied to the TSK-DEAE column. Solvent A was 10 m*M* Tris–acetic acid and solvent B was 0.5 *M* Tris–acetic acid (both pH 7.2). The column was eluted with: 0 to 20% B over 10 min, 20% B for 4 min, 20 to 100% B over 20 min, flow-rate 0.7 ml/min. Fractions (0.7 ml) were collected for peptidase assay.

For semi-preparative separation, the freeze-dried extract (100 mg) was dissolved in 2 ml of 0.1 *M* Tris–acetic acid (pH 7.2), 2 m*M* dithiothreitol (DTT) and chromatographed on the TSK-DEAE column, eluting with a 30-min gradient from 0.1 to 0.4 *M* Tris–acetic acid containing 2 m*M* DTT at 0.7 ml/min. Further purification of the active fractions was achieved using the same gradient over 150 min at a flow-rate of 0.14 ml/min, followed by gel-filtration on TSK-G4000 as before.

Use of reversed-phase HPLC for assaying peptidase activity

Aliquots of column fractions (5–25 μl, depending on initial activity) were incubated with NT (5 μg) in a total volume of 50 μl of 50 m*M* Tris–acetic acid, 2 m*M* DTT (pH 7.2) for 30 min at 37°C. The reaction was terminated by adding 1% TFA (150 μl). The products were separated on a μBondapak C_{18} radial compression cartridge fitted with a guard column[9], which was eluted with a 10 min gradient from 3 to 70% B at 2 ml/min. Solvent A was 11 m*M* TFA, 2.6 m*M* acetic acid, solvent B was 11 m*M* TFA in 70% acetonitrile. The time from injection to injection on the automated HPLC system was 15 min, allowing a typical ion-exchange or gel-filtration separation to be assayed overnight. The products of NT degradation were identified by their elution time[3,10] and confirmed in the case of NT(1–8)* and NT(9–13) by amino acid analysis[10].

Gel-filtration HPLC

Fractions containing NT-degrading activity from the ion-exchange separation were pooled and concentrated by ultrafiltration (Amicon Model 8MC fitted with YM10 membrane) to 200 μl. This was applied to the TSK G4000SW column, which was eluted with 50 m*M* Tris–acetic acid, 2 m*M* DTT (pH 7.2) at 1 ml/min and fractions (1 or 0.5 ml) were collected for assay of peptidase activity. The V_0 and V_t of the column were determined using Dextran blue and tryptophan.

* Figures in parentheses denote amino acid fragment.

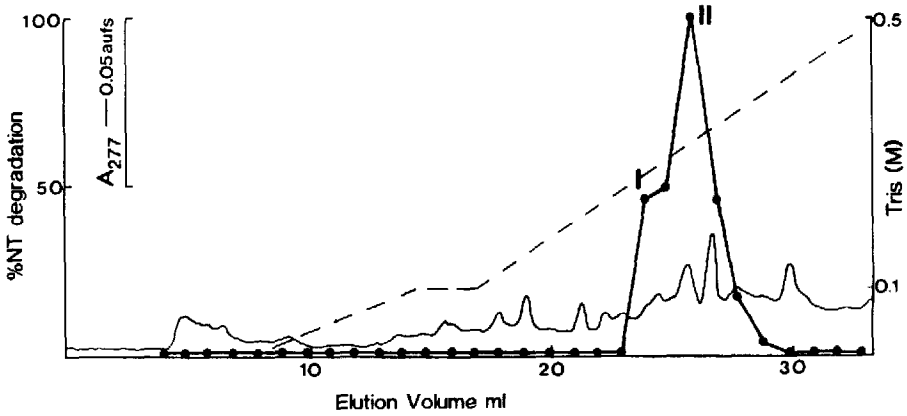

Fig. 2. High-performance ion-exchange chromatography of dialysed brain extract on TSK-545DEAE, eluting with a gradient of increasing Tris–acetic acid molarity at 0.7 ml/min (see Experimental). Neurotensin-degrading activity (●—●) was measured by incubating 25 μl of each fraction with 5 μg of NT in 50 μl total volume for 30 min and measuring NT degradation and product formation by reversed-phase HPLC (see Experimental and Fig. 8).

RESULTS

Analytical separation

Initial chromatography of the brain extract on TSK-DEAE gave a peak of NT-degrading activity preceded by a shoulder (Fig. 2). Using a shallower gradient (0.2–0.4 *M* Tris over 30 min), better resolution of the two peaks was obtained (Fig. 3). The products of NT-degradation by these fractions (Table I) suggest that the peptidase eluted earlier (peak I) is hydrolysing the Pro^{10}–Tyr^{11} bond and that the second peptidase (peak II) is hydrolysing the Arg^{8}–Arg^{9} bond. Peak I was incubated with three other proline-containing peptides (TRH, LH-RH, substance-P), and in each case the Pro–X bonds were hydrolysed. The activity could be inhibited with an antibody to proline endopeptidase[11], indicating that this enzyme (E.C. 3.4.21.26) is responsible for the NT-degrading activity in peak I.

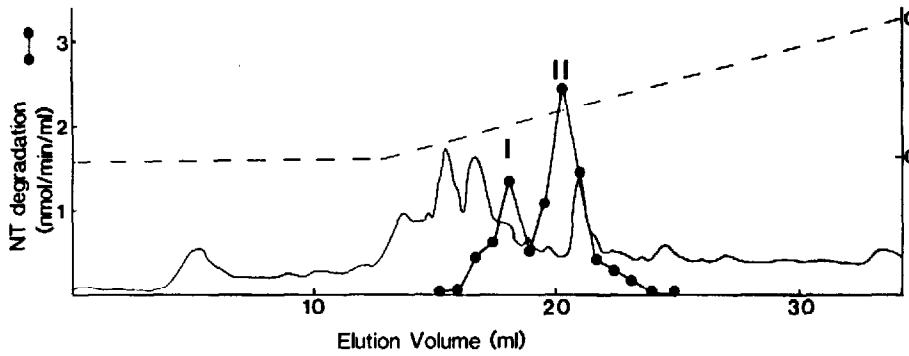

Fig. 3. High-performance ion-exchange chromatography of dialysed brain extract on TSK-545DEAE, eluting with a 0.2–0.4 *M* gradient of Tris–acetic acid (pH 7.2) over 30 min at 0.7 ml/min. Neurotensin-degrading activity (●—●) was measured as in Fig. 2.

TABLE I

PRODUCTS OF NEUROTENSIN DEGRADATION BY PEPTIDASES IN ION-EXCHANGE HPLC FRACTIONS

Fractions containing enzyme activity (Peaks I, II and III from Figs. 2 and 4) were incubated with NT, and the products were separated by reversed-phase HPLC on a Radial-Pak μBondapak C_{18} cartridge (Fig. 8) and identified by amino acid analysis[3,10].

Peak	*Neurotensin products*	
	Brain	*Pituitary*
I	NT(1–10), NT(11–13)	NT(1–10), NT(11–13)
II	NT(1–8), NT(9–13)	NT(1–8), NT(9–13)
III		NT(1–12)

Chromatography of the pituitary extract showed two peaks of activity, eluted at the same place as with the brain extract (Fig. 4). The specificity also appeared to be the same. In addition, a third region of peptidase activity was present which hydrolysed neurotensin at the Ile^{12}–Leu^{13} bond. Little further work on this peptidase has yet been carried out, beyond demonstrating that it can be inhibited by thiol-blocking reagents (*e.g.* 2 m*M* *p*-chloromercuribenzoate).

The fractions containing the peptidase which hydrolyses Arg^8–Arg^9 of NT were pooled, concentrated, and subjected to chromatography on TSK-G4000 SW. Both the brain and pituitary gave a single peak of activity (Fig. 5) with V_e = 20.2 ml. Rat albumin was eluted at 20.3 ml, suggesting that the peptidase has a molecular weight of about 65,000–70,000.

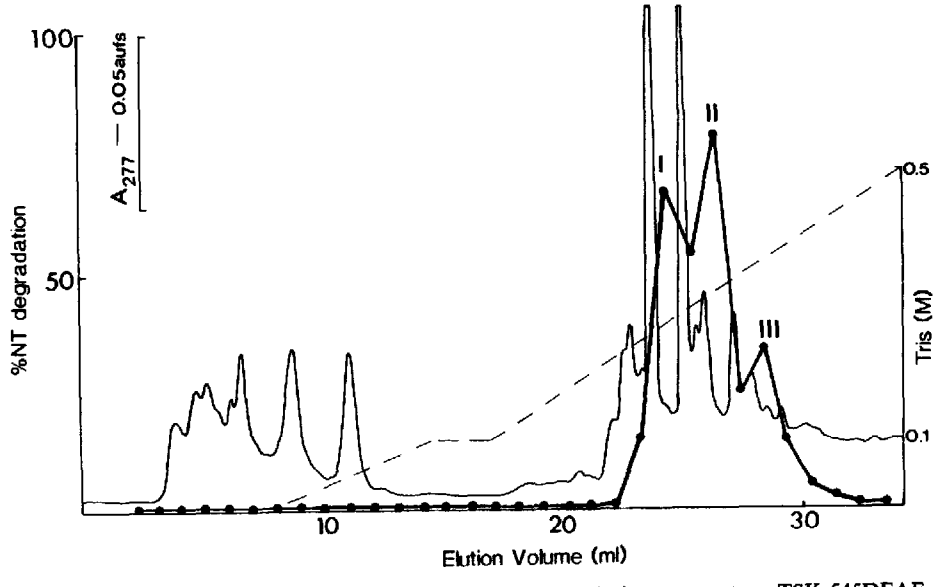

Fig. 4. High-performance ion-exchange chromatography of pituitary extract on TSK-545DEAE, as in Fig. 2. The products of neurotensin degradation by peaks I, II and III are shown in Table I.

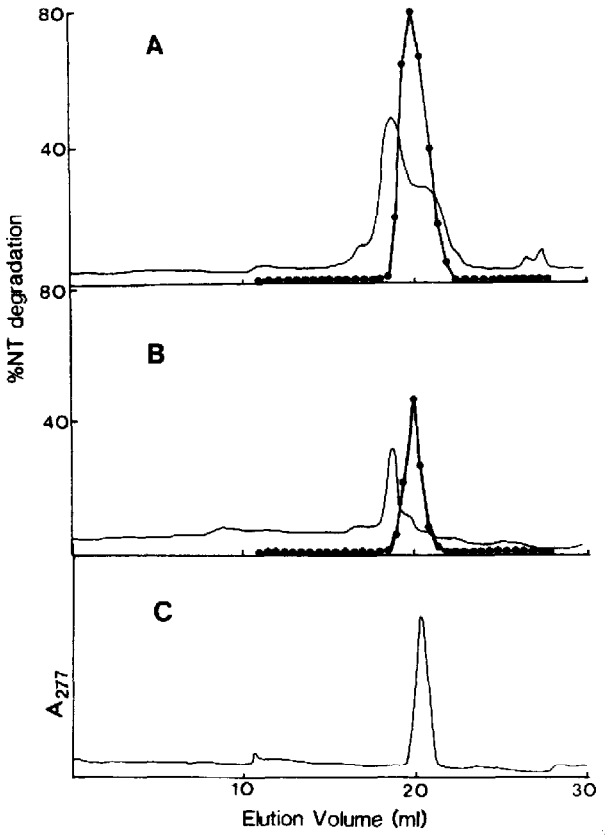

Fig. 5. High-performance gel-filtration chromatography of the major neurotensin-degrading enzyme (AACE) on TSK-G4000SW. A, brain; B, pituitary; C, rat albumin. Fractions from ion-exchange HPLC containing the peptidase activity which hydrolyses NT at Arg^8–Arg^9 were pooled (peak II, Figs. 2 and 4) and concentrated to 200 μl by ultrafiltration before injection into the column. Flow-rate 1 ml/min, 0.2 a.u.f.s.

Semi-preparative separations

The NT-degrading activity in the brain extract could be conveniently concentrated by adsorption on DEAE-cellulose, followed by elution with 0.35 *M* Tris. Chromatography of 100 mg of the dialysed, freeze-dried 0.35 *M* Tris eluate on TSK-DEAE showed a broad area of relatively poorly resolved UV-absorbing material (Fig. 6). AACE activity was eluted at the same molarity of Tris as in the analytical separations. Although no distinct peak of proline endopeptidase activity was apparent, the products of NT-degradation indicated that this enzyme was present in the first two fractions containing peptidase activity. Further purification of the active fractions on TSK-DEAE at a reduced flow-rate was followed by chromatography on TSK-G4000. Analysis of the purified enzyme on TSK-DEAE and TSK-G4000 showed a single UV peak coinciding with the enzyme activity (Fig. 7). No activity due to proline endopeptidase (peak I) could be detected in the purified enzyme with a sensitive fluorogenic assay. The fractions containing the main AACE activity were pooled, divided into aliquots and freeze dried. The enzyme was stable for up to three

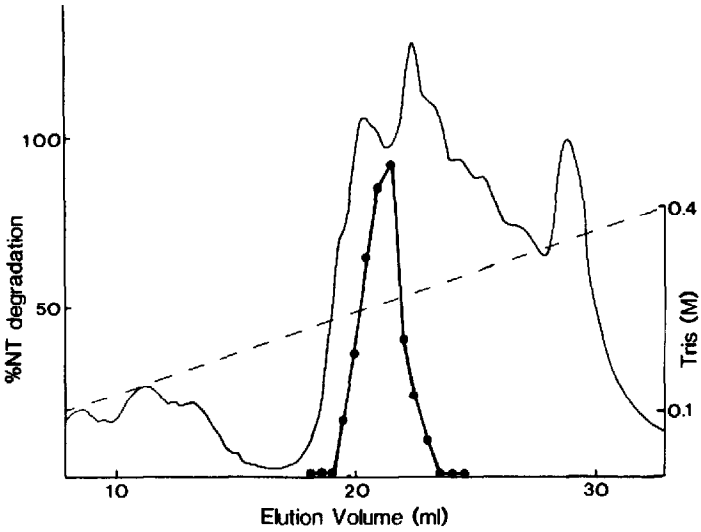

Fig. 6. High-performance ion-exchange chromatography (TSK-545DEAE) of 100 mg of dialysed, freeze-dried brain extract after batch purification on DEAE-cellulose (see Experimental). 0.1–0.4 *M* Tris–acetic acid (pH 7.2) gradient over 30 min, 0.7 ml/min, 0.5 a.u.f.s. at 277 nm. Neurotensin-degrading activity (●—●) was assayed in 5 μl of the fraction (see Experimental). The four fractions with highest activity were pooled for further purification.

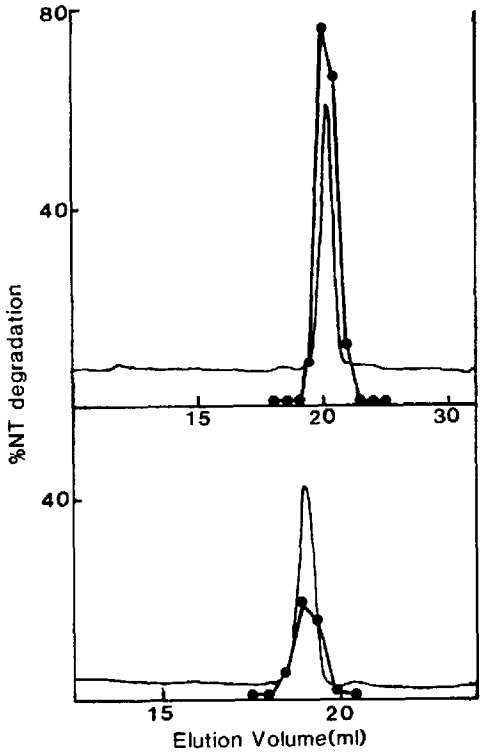

Fig. 7. Analysis of purity of the major NT-degrading enzyme using TSK-G4000SW (upper trace) eluted with 0.1 *M* Tris–acetic acid (pH 7.2), 2 m*M* DTT (0.5 ml/min) and TSK-545DEAE (lower trace) eluted with a 30 min gradient of 0.1–0.4 *M* Tris-acetic acid (pH 7.2) containing 2 m*M* DTT at 0.7 ml/min. 0.02 a.u.f.s. at 277 nm.

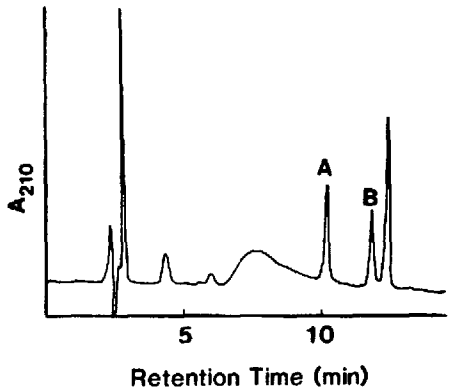

Fig. 8. Reversed-phase HPLC of the products of neurotensin degradation by purified AACE, using a Radial-Pak cartridge (μBondapak C_{18}) in the Z-module compression unit (see Experimental). Amino acid analysis of the peaks (Table II) shows that A is NT(1–8) and B is NT(9–13). The peak eluted just after peak B is unchanged neurotensin. Peaks eluted earlier than A were present in the reagent blank.

TABLE II

AMINO ACID COMPOSITION OF NEUROTENSIN DEGRADATION PRODUCTS

Products of NT degradation by the purified major enzyme from brain (peak I, Fig. 1) were separated by reversed-phase HPLC (Fig. 8), hydrolysed (110°C. 18 h), and analysed on a Rank Hilger Chromaspek amino acid analyser.

Peak	*Amino acid composition (residues/molecule of peptide)*									
	nmol	*Asp*	*Gly*	*Pro*	*Ile*	*Leu*	*Tyr*	*Lys*	*Arg*	*Assignment*
A	1.8	1.06	2.12	0.89	–	1.06	0.89	1.01	0.95	NT(1–8)
B	1.7	–	–	1.06	0.88	1.00	1.12	–	0.94	NT(9–13)

months in this form and could be reconstituted for further characterization studies without loss of activity. Amino acid analysis of the products of NT-degradation by AACE confirm that the enzyme hydrolyses the Arg^8–Arg^9 bond, giving rise to equimolar amounts of NT(1–8) and NT(9–13) (Fig. 8 Table II). Subsequent studies have shown that it is thiol-dependent peptidase with a K_m of 43 μM and a pH optimum of 7.5[13].

CONCLUSIONS

Reversed-phase HPLC has proved invaluable in studying the metabolism of neuropeptides by CNS peptidases[2,3,10,12]. The present work demonstrates the added usefulness of ion-exchange and gel-filtration HPLC on TSK-GEL type SW column materials for the identification and isolation of neuropeptide-metabolizing enzymes using neurotensin as the example. The major advantage over conventional chromatographic techniques is in the speed with which separations can be achieved, particularly important for isolating sensitive biologically active materials. Other advan-

tages are that resolution, recoveries and sensitivity are all high and that chromatography is carried out in neutral aqueous buffers. Gradients of increasing Tris molarity can be used to avoid high sodium chloride concentrations.

An important factor in this study was the ability to assay rapidly the peptidase activity in column fractions by automated reversed-phase HPLC on radial compression cartridges. In this way, the degradation products could be identified, and the bond specificity of the enzyme activities could thus be determined. In fact, significantly shorter analysis times than those achieved here are possible under fully optimized conditions. The use of chromogenic substrates for identifying peptidase activity is limited when searching for uncharacterized enzymes, whereas the use of peptide radioimmunoassay (RIA) to follow the degradation of the peptide is prone to errors since metabolites of the peptide may cross-react in the RIA.

Of the three readily soluble NT-degrading enzymes identified in brain and pituitary extracts, the major one was found to be an endopeptidase cleaving NT at Arg^8–Arg^9 (AACE). The enzyme preparation obtained by semi-preparative separation using analytical TSK-GEL ion-exchange and gel-filtration columns was substantially free from peptidase activity against a wide range of neuropeptides under conditions where 90% of NT was degraded[13]. The dynorphin fragment (amino acids 1–8), which also contains the sequence Arg–Arg, is the only other substrate which approaches NT in its rate of degradation. While the enzyme activity was eluted as a single UV-absorbing peak on both TSK-DEAE and TSK-G4000, its purity needs to be established by independent criteria. Further studies will concentrate on the role of this enzyme in NT metabolism in vivo and its relationship to membrane-bound NT-degrading activity[3].

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