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Studies on Peptides. CV.^{1,2)} Synthesis of Chicken Neurotensin

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A tridecapeptide corresponding to the entire amino acid sequence of chicken neurotensin was synthesized by applying a new deprotecting procedure with trifluoromethanesulfonic acid-thioanisole in combination with a new arginine derivative, Arg(mesitylene-2-sulfonyl). The synthetic avian neurotensin was found to be as active as synthetic bovine neurotensin in terms of contractile activity in isolated guinea-pig ileum and hypotensive effects in rats.

Keywords—chicken intestinal peptide; conventional synthesis of chicken neurotensin; mesitylene-2-sulfonylarginine; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; HPLC of synthetic chicken neurotensin

Recently, Carraway and Bhatnagar³⁾ elucidated the structure of a tridecapeptide (I) isolated from chicken intestine. The amino acid sequence of this new peptide closely resembles that of hitherto known mammalian neurotensin.⁴⁾ Structural difference between the two peptides has been noted in the locations of three amino acid residues. Histidine, valine and alanine are located at positions 3, 4 and 7 in chicken neurotensin respectively, while tyrosine, glutamic acid and proline occupy these positions in the latter.

We wish to report the synthesis of the tridecapeptide corresponding to the entire amino acid sequence of chicken neurotensin, as shown in Fig. 1. The C-terminal hexapeptide, Z(OMe)-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl, used for our previous synthesis of bovine neurotensin,⁵⁾ served as the starting material for the present synthesis of this newly found avian intestinal peptide.

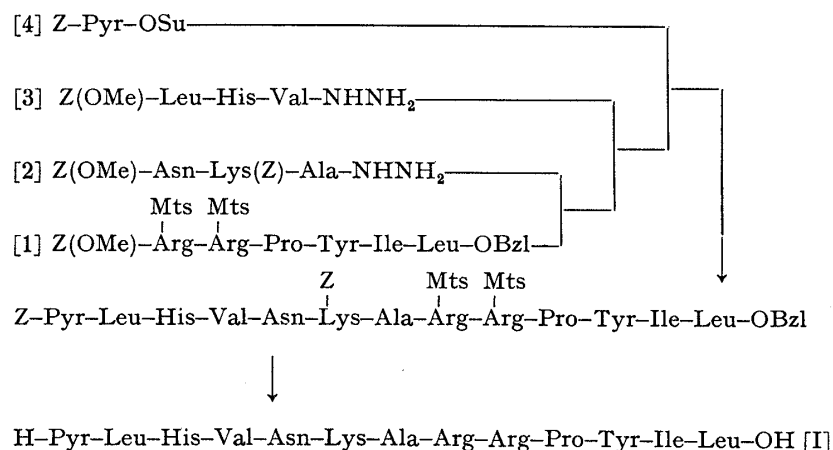


Fig. 1. Synthetic Scheme for Chicken Neurotensin

Two fragments, Z(OMe)-Asn-Lys(Z)-Ala-NHNH₂ and Z(OMe)-Leu-His-Val-NHNH₂, were newly synthesized. The former was synthesized by condensation of Z(OMe)-Asn-OH with a TFA-treated sample of Z(OMe)-Lys(Z)-Ala-OMe⁶⁾ by the NP method,⁷⁾ followed by

exposure of the resulting protected tripeptide ester, Z(OMe)-Asn-Lys(Z)-Ala-OMe, to hydrazine hydrate as usual. For the synthesis of the latter hydrazide, Z(OMe)-Leu-His-OMe was first prepared by the mixed anhydride procedure,⁸⁾ instead of the DCC procedure in order to avoid DCC adduct formation at the His residue.⁹⁾ The resulting protected dipeptide ester was converted to the corresponding hydrazide and subsequently coupled with H-Val-OMe *via* the azide¹⁰⁾ without particular difficulty. Conversion of the resulting protected tripeptide ester, Z(OMe)-Leu-His-Val-OMe, to the corresponding hydrazide required somewhat longer treatment with hydrazine hydrate than usual, presumably due to the steric hindrance of the Val side chain.

Successive azide condensations of these two fragments onto a TFA-treated sample of the above hexapeptide ester were performed using 1.5 equivalents of the acyl component in each step. From the ninhydrin-negative reaction mixtures the desired protected peptides were isolated in satisfactory yields in both cases. Silica gel chromatography was effective for purification of these protected intermediates. The final condensation of Z-Pyr-OSu was conducted until the reaction mixture became ninhydrin-negative and the desired protected tridecapeptide ester, Z-Pyr-Leu-His-Val-Asn-Lys(Z)-Ala-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl, was easily purified by precipitation from DMF with ethyl acetate.

The deprotection in the final step of the synthesis was performed in two ways, by HF¹¹⁾ and by 1 M TFMSA-thioanisole in TFA.¹²⁾ Especially in the latter instance, the treatment at 0°C for 120 min was repeated three times to ensure complete deprotection. Each deprotected product was purified by ion-exchange chromatography on CM-cellulose followed by partition chromatography on Sephadex G-25¹³⁾ to afford a homogeneous product as examined by TLC and HPLC. The yield was 39% in the HF deprotection and 42% in the latter thioanisole-mediated deprotection. This synthesis provided additional evidence of the usefulness of the new TFMSA deprotecting procedure and the new arginine derivative, Arg(Mts).¹⁴⁾

When contractile activity in isolated guinea-pig ileum was examined, the relative potency of synthetic avian neurotensin was 1.27 ± 0.12 with respect to that of synthetic bovine neurotensin. Our synthetic avian neurotensin was also as active as synthetic bovine neurotensin, in terms of hypotensive effects in rats. In the latter assay, no tachyphylaxis was observed.

Experimental

General experimental methods employed in this investigation are essentially the same as described in Part 87¹⁵⁾ of this series. *R_f* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: *R_{f1}* CHCl₃-MeOH-H₂O (8:3:1), *R_{f2}* *n*-BuOH-AcOH-pyridine-H₂O (30:6:20:24), *R_{f3}* *n*-BuOH-AcOH-pyridine-H₂O (4:1:1:2). HPLC was conducted with a Waters 204 Compact Model, using a column of Cosmosil (5C18) (4.6 × 150 mm) with gradient elution from 40% MeOH to 60% MeOH.

Z(OMe)-Asn-Lys(Z)-Ala-OMe-Z(OMe)—Lys(Z)-Ala-OMe (8.90 g, 16.81 mmol) was treated with TFA-anisole (22 ml–2.3 ml) in an ice-bath for 60 min, then dry *n*-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 4 h and dissolved in DMF (7 ml), together with Et₃N (5.1 ml, 37.0 mmol) and Z(OMe)-Asn-ONP (8.42 g, 20.17 mmol). After being stirred at room temperature for 24 h, the mixture was concentrated and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H₂O and precipitated twice from DMF with MeOH; yield 6.97 g (65%), mp 202–204°C, $[\alpha]_D^{20} -14.1^\circ$ ($c=1.0$, DMF), *R_{f1}* 0.69. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.05, Ala 1.00, Lys 1.02 (recovery of Ala, 89%). *Anal.* Calcd for C₃₁H₄₁N₅O₁₀: C, 57.84; H, 6.42; N, 10.88. Found: C, 57.89; H, 6.36; N, 10.75.

Z(OMe)-Asn-Lys(Z)-Ala-NHNH₂ [2]—Z(OMe)-Asn-Lys(Z)-Ala-OMe (7.0 g, 10.88 mmol) in DMF-MeOH (20 ml–5 ml) was treated with 80% hydrazine hydrate (3.4 ml, 5 eq) at room temperature overnight. The resulting gelatinous product was collected by filtration, washed with MeOH and precipitated from DMF with MeOH; yield 6.66 g (95%), mp 217–220°C, $[\alpha]_D^{20} -4.1^\circ$ ($c=1.0$, DMF), *R_{f1}* 0.50. *Anal.* Calcd for C₃₀H₄₁N₇O₉·H₂O: C, 54.45; H, 6.55; N, 14.82. Found: C, 54.45; H, 6.46; N, 14.53.

Z(OMe)-Leu-His-OMe—A mixed anhydride [prepared from 7.01 g (25.18 mmol) of Z(OMe)-Leu-OH as usual] in THF (20 ml) was added to an ice-chilled solution of H-His-OMe [prepared from 5.29 g (25.18 mmol) of the dihydrochloride] in DMF (15 ml) and the mixture was stirred in an ice-bath for 2 h. After removal of the solvent by evaporation, the residue was dissolved in AcOEt. The extract was washed with 5% NaHCO₃ and H₂O, dried over Na₂SO₄ and concentrated. The residue was recrystallized twice from MeOH and ether;

yield 8.80 g (82%), mp 120—122°C, $[\alpha]_D^{20} -16.9^\circ$ ($c=1.0$, MeOH), R_f 0.75. *Anal.* Calcd for $C_{22}H_{30}N_4O_6$: C, 59.18; H, 6.77; N, 12.55. Found: C, 59.21; H, 6.74; N, 12.47.

Z(OMe)-Leu-His-NHNH₂—Z(OMe)-Leu-His-OMe (7.50 g, 16.80 mmol) in MeOH (35 ml) was treated with 80% hydrazine hydrate (5.3 ml, 5 eq) overnight. The solvent was evaporated off and the residue was recrystallized from MeOH and ether; yield 7.16 g (95%), mp 203—205°C, $[\alpha]_D^{20} -36.9^\circ$ ($c=1.0$, MeOH), R_f 0.55. Amino acid ratios in 6 N HCl hydrolysate: Leu 1.00, His 1.07 (recovery of Leu, 94%). *Anal.* Calcd for $C_{21}H_{30}N_6O_5$: C, 56.49; H, 6.77; N, 18.82. Found: C, 56.76; H, 6.93; N, 18.89.

Z(OMe)-Leu-His-Val-OMe—The azide [prepared from 8.01 g (17.92 mmol) of Z(OMe)-Leu-His-NHNH₂] in DMF (15 ml) and Et₃N (2.47 ml, 17.92 mmol) were added to an ice-chilled solution of H-Val-OMe [prepared from 3.60 g, (21.48 mmol) of the hydrochloride] in DMF (15 ml) and the mixture was stirred at 4°C for 24 h. The solvent was evaporated off and the residue was treated with H₂O. The resulting powder was washed with 5% NaHCO₃ and H₂O and then recrystallized from MeOH and ether; yield 5.75 g (59%), mp 210—212°C, $[\alpha]_D^{20} -40.0^\circ$ ($c=1.0$, MeOH). R_f 0.58. *Anal.* Calcd for $C_{27}H_{39}N_5O_7$: C, 59.43; H, 7.20; N, 12.84. Found: C, 59.44; H, 7.28; N, 12.86.

Z(OMe)-Leu-His-Val-NHNH₂ [3]—Z(OMe)-Leu-His-Val-OMe (5.13 g, 9.40 mmol) dissolved in MeOH (18 ml) was treated with 80% hydrazine hydrate (3.0 ml, 5 eq) at room temperature overnight. After further addition of 80% hydrazine hydrate (3.0 ml, 5 eq), the solution was warmed at 40—50°C for 60 min and then kept standing at room temperature overnight. The resulting gelatinous product was collected by filtration and recrystallized three times from MeOH; yield 4.75 g (93%), mp 246—249°C, $[\alpha]_D^{20} -43.5^\circ$ ($c=1.0$, MeOH). R_f 0.49. Amino acid ratios in 6 N HCl hydrolysate: Leu 1.00, Val 0.98, His 1.02 (recovery of Leu, 91%). *Anal.* Calcd for $C_{26}H_{39}N_7O_6 \cdot 1/2H_2O$: C, 56.30; H, 7.27; N, 17.68. Found: C, 56.23; H, 7.09; N, 17.54.

Z(OMe)-Asn-Lys(Z)-Ala-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl—Z(OMe)-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl (1.30 g, 0.91 mmol) was treated with TFA-anisole (2.5 ml–0.49 ml) as usual then dry ether was added. Trituration of the residue with ether afforded a powder, which was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (5 ml) containing Et₃N (0.13 ml, 0.91 mmol). The azide [prepared from 0.88 g (1.37 mmol) of Z(OMe)-Asn-Lys(Z)-Ala-NHNH₂] in DMF (1.5 ml) and Et₃N (0.19 ml, 1.37 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 18 h. The solvent was evaporated off and the residue was treated with 5% citric acid. The resulting powder was recrystallized from MeOH and ether and then applied to a column of silica gel (2.0 × 11.0 cm), which was eluted with CHCl₃-MeOH (9:1, v/v). The fractions containing the substance of R_f 0.62 were combined, the solvent was removed by evaporation and the residue was recrystallized from MeOH and ether; yield 1.23 g (72%), mp 139—140°C, $[\alpha]_D^{20} -32.7^\circ$ ($c=1.0$, MeOH), R_f 0.62. Amino acid ratios in 6 N HCl (with phenol) hydrolysate: Asp 1.14, Pro 1.04, Ala 1.05, Ile 0.98, Leu 1.00, Tyr 0.97, Lys 1.26, Arg 2.10 (recovery of Leu, 94%). *Anal.* Calcd for $C_{93}H_{127}N_{17}O_{21}S_2 \cdot 1.5H_2O$: C, 58.47; H, 6.86; N, 12.47. Found: C, 58.17; H, 6.70; N, 12.81.

Z(OMe)-Leu-His-Val-Asn-Lys(Z)-Ala-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl—The above protected nonapeptide ester (1.03 g, 0.53 mmol) was treated with TFA-anisole (1.4 ml–0.29 ml) and the N α -deprotected peptide isolated as stated above was dissolved in DMF (2.5 ml) containing Et₃N (0.07 ml, 0.53 mmol). The azide [prepared from 0.43 g (0.80 mmol) of Z(OMe)-Leu-His-Val-NHNH₂] in DMF (1.0 ml) and Et₃N (0.11 ml, 0.80 mmol) were added and the mixture was stirred at 4°C for 24 h. After removal of the solvent by evaporation, the residue was treated with 5% NaHCO₃. The resulting powder was washed with H₂O and precipitated from DMF with ether. The product was then dissolved in a small amount of CHCl₃-H₂O-DMF (9:1:0.5, v/v) and applied to a column of silica gel (2.0 × 11.0 cm) equilibrated previously with CHCl₃-H₂O (9:1, v/v) and the fractions containing the substance of R_f 0.60 were combined. The solvent was removed by evaporation and the residue was triturated with H₂O to afford a powder, which was precipitated from DMF with ether; yield 725 mg (61%), mp 185—187°C, $[\alpha]_D^{20} -15.7^\circ$ ($c=0.5$, DMF), R_f 0.60. Amino acid ratios in 6 N HCl (with phenol) hydrolysate: Asp 1.02, Pro 1.02, Ala 0.99, Val 1.00, Ile 0.90, Leu 2.00, Tyr 0.90, Lys 1.13, His 1.13, Arg 2.10 (recovery of Leu, 93%). *Anal.* Calcd for $C_{110}H_{154}N_{22}O_{24}S_2 \cdot 1.5H_2O$: C, 58.46; H, 7.00; N, 13.64. Found: C, 58.22; H, 6.95; N, 13.76.

Z-Pyr-Leu-His-Val-Asn-Lys(Z)-Ala-Arg(Mts)-Arg(Mts)-Pro-Tyr-Ile-Leu-OBzl—The above protected dodecapeptide ester (685 mg, 0.30 mmol) was treated with TFA-anisole (0.8 ml–0.16 ml) and the N α -deprotected peptide isolated as stated above was dissolved in DMF (1.0 ml), together with Et₃N (0.12 ml, 0.90 mmol) and Z-Pyr-OSu (216 mg, 0.60 mmol). After being stirred at room temperature for 24 h, the mixture was concentrated and the residue was treated with 5% NaHCO₃ to afford a powder, which was washed with 5% NaHCO₃, 0.3% AcOH and H₂O and precipitated three times from DMF with AcOEt; yield 520 mg (75%), mp 213—215°C, $[\alpha]_D^{20} -33.0^\circ$ ($c=0.7$, DMF), R_f 0.55. Amino acid ratios in 6 N HCl (with phenol) hydrolysate: Asp 1.03, Glu 1.10, Pro 0.96, Ala 0.96, Val 1.00, Ile 0.92, Leu 2.00, Tyr 0.90, Lys 1.09, His 1.00, Arg 1.90 (recovery of Leu 90%). *Anal.* Calcd for $C_{114}H_{157}N_{23}O_{25}S_2 \cdot 3H_2O$: C, 57.82; H, 6.94; N, 13.61. Found: C, 57.79; H, 6.74; N, 13.30.

H-Pyr-Leu-His-Val-Asn-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu-OH (Chicken Neurotensin)—a) TFMSA deprotection: The protected tridecapeptide ester (71.2 mg, 30.8 μ mol) was treated with 1 M TFMSA-thioanisole in TFA (1.5 ml, 50 eq) in the presence of *m*-cresol (0.03 ml, 100 eq) in an ice-bath for 120 min,

then dry ether was added. This treatment was repeated under identical conditions and the resulting product was dissolved in H_2O (10 ml) and stirred with Amberlite CG-4B (acetate form, approximately 0.5 g) for 30 min. After filtration followed by lyophilization, the resulting powder was dissolved in a small amount of H_2O and applied to a column of CM-cellulose (1.1×9.0 cm), which was eluted with 0.2 M NH_4HCO_3 , pH 7.9 (100 ml), through a mixing flask containing H_2O (50 ml). The UV absorption at 275 nm was determined in each fraction (2 ml). The presence of three peaks (Fig. 2-a, line i) indicated incomplete deprotection under the conditions employed above. The fractions corresponding to all of these peaks were combined and lyophilized. The resulting powder was again treated with the same deprotecting reagents as stated above. After conversion to the corresponding acetate, the re-treated product was purified by column chromatography on CM-cellulose under identical conditions. There was a small peak in the front eluate, and a main peak (Fig. 2-a, line ii) from which (Tube No. 20–26), after lyophilization, a fluffy powder was obtained; yield 35.5 mg (73%). Single spot on TLC, R_f 0.51. HPLC examination revealed the presence of at least three minor impurities as shown in Fig. 3-i. The CM-purified product was then subjected to partition chromatography on Sephadex G-25 (1.6×27.5 cm) previously equilibrated with the lower solution of n -BuOH-AcOH- H_2O (4:1:5). The column was then eluted with the upper solution of the above solvent system and the UV absorption at 275 nm was determined in each fraction (2 ml). After elution of a small peak, the desired compound was eluted with the lower solution (100 ml). Lyophilization of the main fraction (Fig. 4, tube No. 43–46) gave a white fluffy powder; yield 20.4 mg (42%); $[\alpha]_D^{25} -93.2^\circ$ ($c=0.5$, H_2O), single spot on TLC: R_f 0.51, R_f 0.44. Single peak on HPLC: retention time, 3.24 min (Fig. 3-ii), calculated purity 98.6%. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Glu 1.09, Pro 1.02, Ala 0.96, Val 1.00, Ile 0.98, Leu 2.00, Tyr 1.01, Lys 1.12, His 1.00, Arg 2.08 (recovery of Leu 91%). Because of the presence of Pyr at the N-terminal, leucine-aminopeptidase digestion was abandoned. Anal. Calcd for $\text{C}_{73}\text{H}_{117}\text{N}_{23}\text{O}_{16} \cdot 3\text{CH}_3\text{COOH} \cdot 5\text{H}_2\text{O}$: C, 51.48; H, 7.60; N, 17.48. Found: C, 51.00; H, 6.98; N, 17.38.

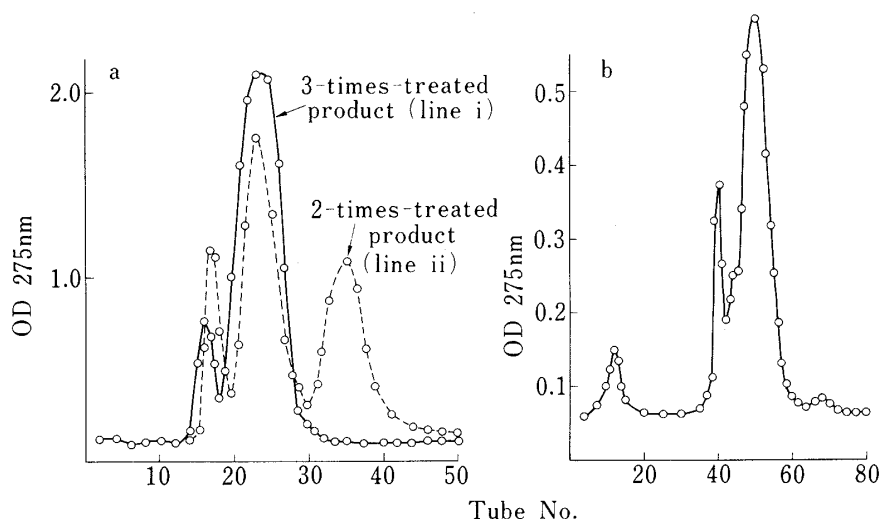


Fig. 2. Purification of Synthetic Chicken Neurotensin by Ion-exchange Chromatography on CM-cellulose

a: TFMSA-protected product

b: HF-protected product

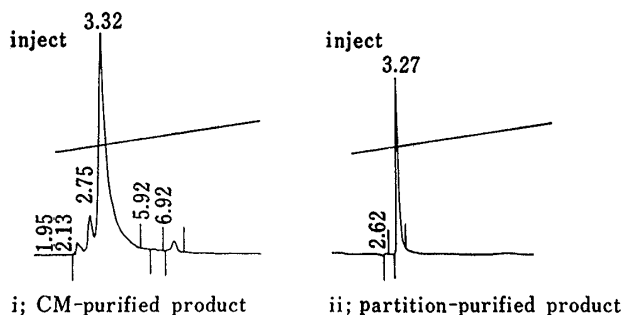


Fig. 3. HPLC Examination of Synthetic Chicken Neurotensin

b) The protected tridecapeptide ester (50.3 mg, 21.7 μ mol) was treated with HF (approximately 3 ml) in the presence of *m*-cresol (0.28 ml, 125 μ eq) in an ice-bath for 45 min, then HF was removed by evaporation and the residue was treated with ether. The deprotected peptide, after conversion to the corresponding acetate, was purified by column chromatography on CM-cellulose (1.5 $\times$ 10 cm) as described above. The column was eluted with 0.2 M NH_4HCO_3 (100 ml), pH 7.9 through a mixing flask containing H_2O (30 ml). The UV absorption at 275 nm was determined in each fraction (3 ml). A main peak with a small shoulder in the front was detected (Fig. 2-b). The main fractions (Tube No. 46—56) were lyophilized to give a fluffy powder; yield 15.5 mg (45%). The CM-purified product was further purified by partition chromatography on Sephadex G-25 (1.6 $\times$ 27.5 cm) as described above. A similar elution pattern was obtained and lyophilization of the main peak (tube No. 46—50 in the lower eluates) afforded a white fluffy powder; yield 13.4 mg (39%). $[\alpha]_D^{25} - 92.5^\circ$ ($c=0.4$, H_2O), R_f 0.51, R_f 0.44, single peak on HPLC, and its retention time (3.24 min) was identical with that of the TFMSA-deprotected product. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.05, Glu 1.05, Pro 1.02, Ala 1.00, Val 0.96, Ile 1.01, Leu 2.00, Tyr 0.99, Lys 1.18, His 1.00, Arg 2.01 (recovery of Leu 95%).

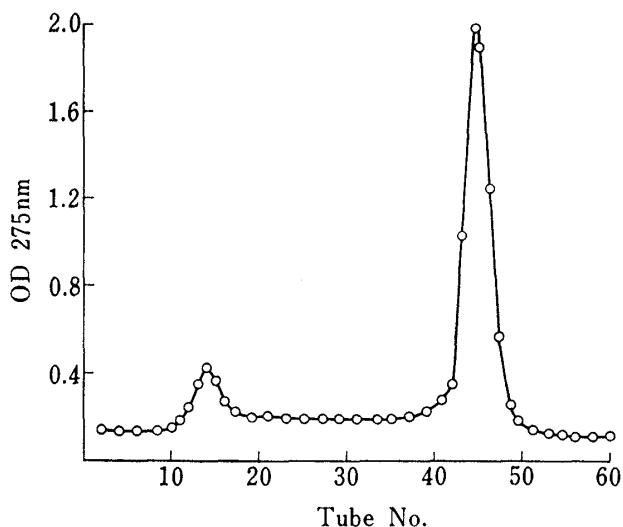


Fig. 4. Purification of CM-purified Chicken Neurotensin by Partition Chromatography on Sephadex G-25

References and Notes

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- 2) Amino acids, peptides and their derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, NP=*p*-nitrophenyl, OSu=N-hydroxysuccinimide ester, DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, DMF=dimethylformamide, TFMSA=trifluoromethanesulfonic acid.
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