

Characterization of Two Synaptosomal Peptides in Calf Brain

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Two low-molecular weight peptides occurring in calf brain nerve terminals and their subcellular vesicles were purified by ion-exchange and thin-layer chromatography, adsorption chromatography on copper-Sephadex and gel filtration. Their amino acid composition and sequence were determined using the dansyl chloride method and carboxypeptidase and aminopeptidase. The tentative sequences NH_2 -alanyl-glycyl-glutamyl-phosphoserine-COOH and *N*-acetylaspartyl-glutamyl-aurine- SO_3 were obtained. Release of both peptides from synaptic vesicles was caused by depolarizing concentrations of K^+ and Ca^{2+} and also by electrical stimulation, but no release from the synaptosomes could be observed. Vesicular origin of the peptides was shown by gel filtration.

The synaptic vesicle fraction of mammalian cerebral cortex contains eight to ten different amino acids¹⁻³ and also certain low-molecular weight peptides. Cuello *et al.*⁴ and Emson *et al.*⁵ have demonstrated that substance P and vasoactive intestinal polypeptide (VIP) are both located in rat synaptic vesicles, and we have earlier demonstrated the presence of certain acidic, low-molecular weight peptides in calf brain synaptic vesicles and synaptoplasm, yielding mainly aspartic acid, glutamic acid, serine, alanine and glycine after acid hydrolysis.^{2,3} The main peptide fraction, eluted just before taurine in ion-exchange chromatography, had a concentration of about 23 $\mu\text{mol/g}$ protein in alanine equivalents, exceeding that of all the other amino compounds in the vesicles.³ The cysteic acid peak also contained some acid-labile, hydrolysable material. These acid-labile compounds have now been purified by ion-exchange and thin-layer chromatography, adsorption chromatography on copper-Sephadex and gel filtration, and their amino acid composition and sequence have been determined.

EXPERIMENTAL

Subcellular fractionation. Synaptic vesicles were prepared on a large scale from calf brain cortex by the sucrose gradient centrifugation method of Whittaker *et al.*⁶ as modified by Morgan *et al.*⁷ and treated for amino acid extraction as described earlier.³ The differential centrifugation method of Kadota and Kadota,⁸ as modified by DeLorenzo and Freedman,⁹ was also used. This latter method gave the crude synaptosomal fraction (P_2) which was used in release studies (see below) and also for obtaining soluble synaptoplasm and synaptic vesicles after hypo-osmotic shock. The protein content of the subcellular fractions was determined by the method of Lowry *et al.*¹⁰

Extraction of peptides. The amino acids and peptides were extracted from the washed synaptosomes and vesicles with 5% trichloroacetic acid (TCA),³ which was then removed by shaking with diethyl ether. The soluble synaptoplasm was similarly deproteinized with TCA. The solutions were lyophilized or evaporated to dryness in evacuated tubes at 50 °C and the residue taken up in 0.1 M HCl or distilled water. Some of the samples and purified peptide fractions were hydrolyzed in 6 M HCl for 6 h at 100 °C, the HCl then being removed in an evaporator.

Ion-exchange chromatography. An automatic amino acid analyzer (Hitachi-Perkin-Elmer 034 Liquid Chromatograph) was used for quantitative determination of the amino compounds in the subcellular extracts and their hydrolysates using known standards for the identification of amino acids. It was also used for separation of the unknown peptides from the amino acids, since the peptides studied here were eluted before the most acidic of the amino acids. The peptide fractions were collected for further study.

Thin-layer and paper chromatography. Whatman No. 1 filter papers and silica gel (Kieselgel G) plates of thickness 0.25 mm were used. Two-dimensional chromatograms were developed with 70% ethanol

in water and 75 % phenol in water, dried and sprayed with ninhydrin (15 % solution in 3 M potassium citrate buffer, pH 5.1). The spots were developed for 10 min at 105 °C. In quantitative TLC the spots of the peptides were immediately scraped down, eluted in acetone predried over CaO, centrifuged and the extinction measured in micro-cuvettes at 570 nm. Alanine treated in a similar manner to the peptides was used as a standard.

Gel filtration. The vesicular origin of the amino compounds was confirmed by gel filtration of the vesicle preparations on a Sephadex G-50 column equilibrated with isotonic Krebs-Ringer phosphate medium (pH 7.4). Sephadex G-15 columns were used for the molecular weight determinations, elution taking place with water.

Purification of peptides on copper-Sephadex. The TCA-soluble peptides of the synaptoplasm and synaptic vesicle fraction were separated from the α -amino acids on copper-Sephadex columns.¹¹ A copper-Sephadex complex was first prepared in an alkaline medium and packed in small columns. Lyophilized subcellular extracts were dissolved in 50 mM sodium borate buffer (pH 11.0) and eluted with the same solution. The retention volumes of the α -amino acids and peptides differed markedly, and GABA could be separated from the peptides by collecting small fractions (1.0 ml) in the initial part of the chromatogram. Amino compounds in the fractions were determined with ninhydrin and identified with TLC as above.

Structure studies. Peptides separated from α -amino acids on copper-Sephadex columns and fractionated on TLC plates, scraped down and

dissolved in water and lyophilized were treated with pancreatic carboxypeptidase (Fluka AG, 5 \times cryst.) for sequence determination by incubating at pH 8.5 for 4 h at 37 °C¹² and taking small samples at certain time intervals and applying these to TLC plates. Dipeptidyl aminopeptidase (Cathepsin C, from bovine spleen, Sigma) was similarly used at pH 6.0.¹³ Dansyl derivatives of the *N*-terminal amino acids were prepared according to Gray¹⁴ and analyzed by paper and thin-layer chromatography. In some cases the *N*-acetyl group of *N*-terminal aspartic acid was first hydrolyzed with 2 M HCl¹⁵ and then dansylated as above. Phosphorus in the peptides was analyzed using ammonium molybdate.

Release studies. In order to study the possible release of the peptides from storage vesicles in the nerve terminals, the latter were prepared by the method of Kadota and Kadota,⁸ as modified by DeLorenzo and Freedman.⁹ A P₂ fraction (crude synaptosomal fraction) prepared by the differential centrifugation method was divided into five portions. One portion was used as a control, and its vesicles prepared by the method of Kadota and Kadota,⁸ using a hypo-osmotically ruptured P₂ fraction, and a second was used to study the intravesicular origin of the peptides by passing the vesicles through a Sephadex G-50 column. The third P₂ portion was treated for 15 min in 70 mM KCl solution at 37 °C and the fourth similarly in 70 mM CaCl₂ solution. The last P₂ portion was submitted to electrical stimulation at 37 °C for 20 min.¹⁶ The vesicles from the P₂ fractions were then prepared as above. The amino acids and peptides

Table 1. Amino acid composition of the peptide fractions of calf brain synaptic vesicles described in Fig. 2.^a

Amino acid	Fraction Total extract	Peptide fraction	Peptide B	Peptide A
Aspartic acid	38.6(51) ^b	44.4(89)	2.0(3)	31.7(41)
Glutamic acid	16.9(47)	33.5(41)	19.3(37)	30.2(39)
Serine	28.9(41)	6.8(18)	29.3(90)	—
Alanine	4.8(18)	6.1(14)	22.9(33)	—
Glycine	10.8(24)	9.2(21)	26.5(34)	—
Taurine	+	+	—	38.1(40)
Phosphoserine	—	—	+	+
Cysteic acid	—	+	+	+
Unknown	+	—	+	+
<i>n</i> ^c	8	3	3	6

^a The values for the total extract are calculated from the results of Lähdesmäki and Winter.³ The peptide fraction contains all the ninhydrin-positive compounds between *N*-acetylaspartate and taurine (the latter excluded). ^b Means (\pm SD) are given in mol %; + = traces. ^c Number of determinations.

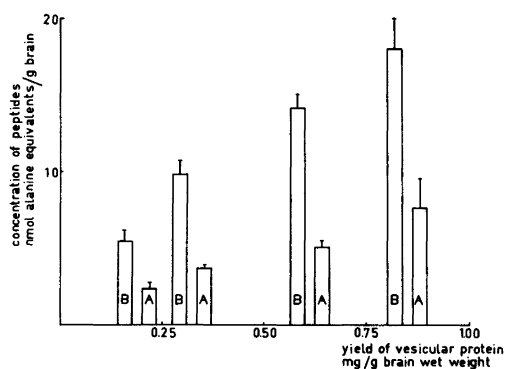


Fig. 1. Concentrations of the peptides A and B (nmol alanine equivalents per 1 g of brain, wet weight) in the calf brain vesicle preparations as a function of protein content. Means (\pm SD) are given from 4–8 experiments.

were extracted with 5% TCA, which was then removed by shaking in diethyl ether, and analyzed on TLC plates as described above. The protein yield of the vesicles prepared by the method of Kadota and Kadota⁸ was 0.24 mg/g brain wet weight.

RESULTS AND DISCUSSION

In addition to ten major amino acids,³ the nerve terminals and their storage vesicles contained two unknown peptides in relatively high concentrations (Table 1; Peptides A and B). They appeared in both the vesicles and the soluble synaptoplasm, their relative amounts being dependent on the purity of the vesicle preparations (Fig. 1). A high purity

preparation gave 6–10 nmol peptide B and 2–3 nmol peptide A in 1 g of brain wet weight and low purity preparations figures which were about twice as great. The protein yield suggests cytoplasmic and membranous contaminants among the vesicles and the figures given above indicate therefore that both peptides also occurred outside the vesicles. A theoretical value for vesicle protein in nerve terminals is approximately 0.18 mg per 1 g of original brain.⁷ The purified vesicles nevertheless contained the same peptides, as confirmed by gel filtration on Sephadex G-50 (Table 2). The vesicles were not retained in the column and were thus completely separated from any extravascular amino acids. The analyses of the amino acids in vesicle preparations purified in this way agreed with those reported earlier,^{2,3} showing only some quantitative loss, chiefly in the case of the cysteic acid peak (Fig. 2).

Peptide A was eluted in ion-exchange chromatography in the same fraction with phosphoserine and cysteic acid, and peptide B just before taurine (Fig. 2). Both peptides (A and B) appeared as single components in two-dimensional thin-layer chromatography on silica gel and in paper chromatography, run in both cases with 70% ethanol in water and 75% phenol in water and stained with ninhydrin (Fig. 3).

Hydrolysis of the total amino acid extract with HCl led to a significant increase in the amounts of aspartic acid, glutamic acid, serine, alanine and glycine (Table 1, cf. Ref. 3). Some of the glutamic acid had originally been in the form of glutamine and certainly some aspartic acid in the form of *N*-acetylaspartate and some serine in that of phosphoserine. Hydrolysis of the peptide fraction obtained from collecting the unknown peaks of the amino

Table 2. Concentrations of peptides A and B in calf brain synaptosomes and synaptic vesicle fraction after various treatments.

Treatment	Synaptosomes (P ₂ fraction)		Synaptic vesicles	
	A nmol/g	B nmol/g	A nmol/g	B nmol/g
Control	5.4(9) ^a	20.2(29)	3.7(5)	9.9(11)
K ⁺ shock (70 mM KCl)	5.3(7)	19.7(33)	1.8(3) ^b	5.0(11) ^b
Ca ²⁺ shock (70 mM CaCl ₂)	5.5(8)	20.4(35)	2.4(3) ^c	8.1(9)
Electrical stimulation	5.1(8)	18.7(31)	2.5(4)	6.9(6) ^c
Gel filtration (Sephadex G-50)	—	—	3.5(4)	9.7(8)

^a Values (means \pm SD) nmol per g brain wet weight in alanine equivalents are from 5 determinations. Significance of differences from control values: ^b $P < 0.01$, ^c $P < 0.05$.

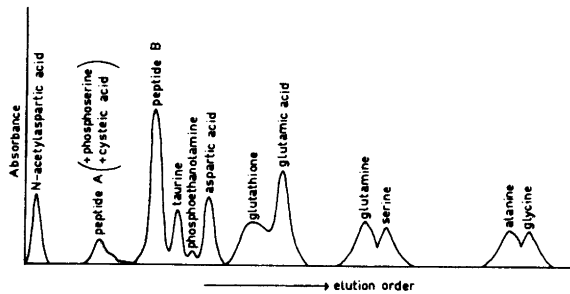


Fig. 2. Initial part of an ion-exchange chromatogram of the amino compounds of calf brain synaptic vesicles from an automatic amino acid analyzer, showing the elution positions of the unknown peptides. The known ninhydrin-positive compounds were identified using standard samples, *N*-acetylaspartate after acid hydrolysis.

acid chromatogram between *N*-acetylaspartate and taurine, liberated the same amino acids as were obtained by hydrolysing the total extract (Table 1). One equivalent of peptide B produced four equivalents of amino acids (glutamic acid, serine, alanine and glycine) and traces of aspartate, phosphoserine and cysteic acid, the latter possibly arising from impurities. Peptide A was hydrolyzed to aspartic acid, glutamic acid and taurine plus some unknown products, the ratios of the main amino acids being about 1:1:1.2.

The synaptosomal peptides could be separated from α -amino acids on copper-Sephadex columns (Fig. 4), and GABA could also be separated from the main peptide fraction by collecting small fractions in the initial part of the chromatogram (Fig. 4).

For structural determination, the peptides were first separated from the amino acids on copper-

Sephadex columns and then fractionated on TLC plates, as described above. The NH_2 -terminal amino acids of the peptides were first determined as dansyl and DNP derivatives. Both reagents gave alanine as the amino-terminal amino acid of the peptide B, but determination of peptide A was difficult as only small amounts of dansyl-aspartate and dansyl-glutamate were obtained. Dansylation became much easier when the peptide samples were first treated with 2 M HCl in a boiling water bath for 30 min. Peptide B then gave dansyl-alanine again, but also dansyl-aspartate and peptide A dansyl-aspartate and small amount of dansyl-glutamate. These results indicate a certain heterogeneity in both peptides, although they appeared as single components in TLC. The major *N*-terminal

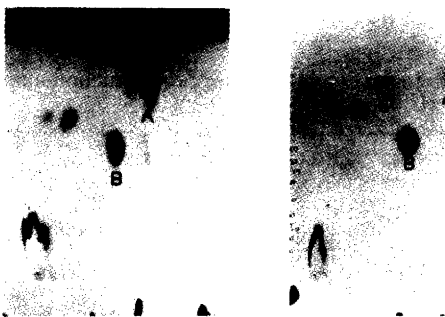


Fig. 3. Thin-layer chromatograms of the peptides A and B in the total vesicle extract (left) and when separated from amino acids on copper-Sephadex (right).

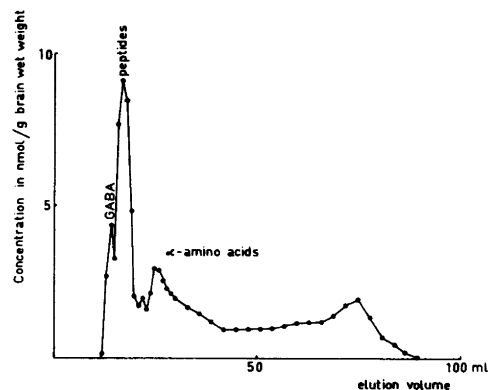


Fig. 4. Chromatography for the separation of α -amino acids and peptides of calf brain synaptic vesicle extract on copper-Sephadex G-25. 0.3 mmol Cu/g Sephadex, pH 11.0, fraction size 1 or 3 ml, elution rate 30 ml/h.

Table 3. Tentative sequences for synaptosomal peptides A and B.^a

A	(<i>N</i> -acetyl)-NH ₂ -aspartyl-glutamyl-taurine-SO ₃ (NH ₂ -aspartyl-taurine-SO ₃) (NH ₂ -glutamyl-taurine-SO ₃)
B	NH ₂ -alanyl-glycyl-glutamyl-(phospho)-serine-COOH (<i>N</i> -acetylaspartyl)-(alanyl)-glycyl-glutamyl-(phospho)-serine-COOH

^a Sequences in parentheses indicate alternative possibilities.

amino acids were alanine for peptide B and *N*-acetylaspartate for peptide A, but peptide A also contained a little aspartate and glutamate as the free NH₂ group of the *N*-terminal amino acids (judging by its reactivity with ninhydrin). Moreover, peptide B also contained some *N*-acetylaspartate as its *N*-terminal amino acid, but for its reactivity with ninhydrin it must mainly contain a free NH₂ group. The relative amounts of the hydrolysis products of peptide A (aspartate:glutamate:taurine = 1:1:1.2) also indicate a certain heterogeneity, and it may contain all three sequences: *N*-acetylaspartyl-glutamyl-taurine, aspartyl-taurine and glutamyl-taurine (Table 3). These tripeptides or dipeptides received a free sulfonic acid group from taurine in all cases, which made them highly acidic.

Peptide B contained almost one equivalent of phosphorus per peptide equivalent. This phosphorus appeared in phosphoserine, which was obtained in addition to serine in the hydrolysates of both HCl and carboxypeptidase.

The peptides A and B were not very good substrates for either carboxypeptidase or dipeptidyl aminopeptidase, as they liberated amino acids from the peptides very slowly. Hydrolysis did take place, however, with an incubation of several hours, and peptide B liberated first, and relatively rapidly, serine and phosphoserine, and then more slowly glutamate and glycine with carboxypeptidase indicating a sequence of this kind at the *C*-terminal end. Dipeptidyl aminopeptidase gave some aspartate and glutamate for peptide A and alanine, glycine and glutamate, in this order, for peptide B. Peptide B was particularly sensitive to elevated temperatures and acids, being partially destroyed in 0.1 M HCl at room temperature, liberating first serine and phosphoserine and then other amino acids (glutamic

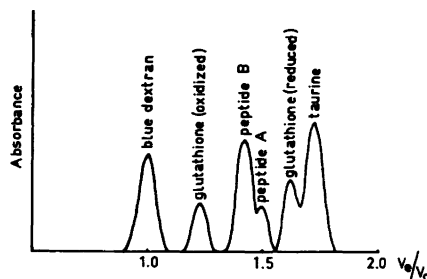


Fig. 5. Gel filtration of peptides A and B together with certain reference substances on a Sephadex G-15 column. Blue dextran was measured at 600 nm and the others at 570 nm after the ninhydrin reaction. Relative absorbances are given as functions of relative elution volumes (V_e/V_0).

acid, glycine and alanine). This liberation order also agreed with that obtained with carboxypeptidase. The data described above are summarized in Table 3 as tentative sequences for the peptides A and B.

Gel filtration of peptides A and B on Sephadex G-15 showed approximate molecular weights of about 400 for peptide A and about 450 for peptide B (Fig. 5), corresponding to tripeptides and tetrapeptides, respectively, and agreeing with those obtained from the sequence determinations. Since any free *N*-acetylaspartate which might have been present originally in the amino acid extract would have been eliminated by ion-exchange and thin-layer chromatography, the peptides identified here possess a mean amino acid composition and sequence quite similar to those reported by Reichelt and Kvamme¹⁷ in mouse brain.

When crude synaptosomal fractions were treated with depolarizing concentrations of KCl and CaCl₂ (70 mM) or submitted to electrical stimulation, the concentrations of both peptides A and B in the synaptic vesicle fraction isolated from the treated synaptosomes decreased significantly (Table 2), but this effect could not be demonstrated in the synaptosomes. This kind of release from synaptic structures is one of the criteria set for transmitters, although the effects of K⁺ and Ca²⁺ ions or electrical stimulation are not very specific. These results together with the observation that these peptides are of an intravesicular origin may support the notion of some important synaptic role.

Results obtained in this laboratory to date thus show: (1) The synaptosomes and synaptic vesicle fraction of calf brain contain high quantities of

certain acidic peptides which are eluted in ion-exchange chromatography in the range of most acidic amino acids. (2) These peptides are soluble in 5% TCA and are thus relatively small molecules. (3) They are hydrolyzed by acid and enzyme, liberating mainly aspartic acid, glutamic acid, serine, alanine, glycine and taurine. (4) They are labile, being partially destroyed at room temperatures and in relatively mild acidic solutions. The isolation and purification procedures for the peptides thus require low temperatures and at most only slightly acidic conditions. (5) They include at least two peptides with molecular weights of approximately 400 and 450, thus being probably tripeptides and tetrapeptides, respectively. (6) The following tentative sequences may be proposed: *N*-Acetylaspartyl-glutamyl-aurine and alanyl-glycyl-glutamyl-phosphoserine, both peptide fractions showing a certain heterogeneity or alternative structures. (7) They are released from synaptic vesicles by depolarizing agents.

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