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# Isolation and sequencing of two cerebellum-specific peptides

**(endogenous peptide analysis/neural markers/synaptosomes)**

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ABSTRACT **By comparing the HPLC elution profiles of peptides isolated from different brain regions, two cerebellum-specific species have been identified. The peptides were isolated by sequential chromatography on reverse phase, followed by ion-pairing HPLC using alkane sulfates as pairing reagents. The sequences of both peptides have been determined by gas-phase Edman degradation and carboxypeptidase Y analysis and were confirmed by synthesis. The larger peptide, termed cerebellin, is a hexadecamer of primary amino acid sequence $NH_2$-Ser-Gly-Ser-Ala-Lys-Val-Ala-Phe-Ser-Ala-Ile-Arg-Ser-Thr-Asn-His-OH. The second peptide is a pentadecamer with a primary sequence identical to residues 2–16 of cerebellin, the nomenclature of which is des-Ser$^1$-cerebellin. Subcellular fractionation of cerebellum followed by ion-pairing HPLC analysis shows that both cerebellin and des-Ser$^1$-cerebellin are enriched between 2.5- and 4-fold in the P2 crude synaptosomal fraction, suggesting their sequestration in some subcellular particle *in vivo*.**

A major goal of developmental neurobiology has been to define the cellular, molecular, and genetic mechanisms that underlie the orderly assembly and maturation of the mammalian nervous system. One fruitful approach to the study of neurodevelopment has been to identify cell-specific markers, usually by immunological techniques, and to assess the expression of these substances in model neural systems (1–5). However, the general procedure of detecting and monitoring marker expression with antibodies does have a number of limitations. First, only antigenic molecules may be studied. Second, immunocytochemical data are often difficult to quantitate. Finally, subtle modifications of the antigenic determinants, such as glycosylation, may result in ambiguous interpretation of data.

In an attempt to circumvent some of the above difficulties, we have tried to characterize brain regions by their native peptide composition. Since the cerebellum has been pointed out to be a particularly useful neural structure for the investigation of the forces regulating neuronal development (6), we have first applied this approach to the rat cerebellum. We report here the isolation and complete primary structures of two related cerebellum-specific peptides that will be shown in further publications to be significant markers for neuronal development in the mammalian cerebellum.

## MATERIALS AND METHODS

**Preparation of Tissue Samples for HPLC Analysis.** Six- to 8-week-old CD, Sprague–Dawley-derived, rats (Charles River Breeding Laboratories) were decapitated, and their brains were rapidly removed, dissected, and immediately frozen in liquid nitrogen. Prior to HPLC analysis, frozen tissues were homogenized in 10 vol of 6 M guanidine hydrochloride using a Polytron (Brinkmann model PT205T probe) at maximum setting for 30 sec at 4°C. The homogenate was diluted with an equal volume of 0.2 M pyridine/1 M acetic acid and then centrifuged at 25,000 × *g* for 30 min. The sample was desalted by passing 20 ml of the supernatant over a 1-ml preparative reversed-phase cartridge (Sep-Pak $C_{18}$, Waters Associates) that had been activated according to the protocol provided by the manufacturers and pre-equilibrated in 0.1% aqueous trifluoroacetic acid by volume. The cartridge was washed once with 20 ml of 0.1% aqueous trifluoroacetic acid and then with 4 ml of 0.1% aqueous trifluoroacetic acid containing 5% (vol/vol) acetonitrile. Peptides were eluted with 4 ml of 0.1% trifluoroacetic acid containing 30% (vol/vol) acetonitrile, this fraction being taken to dryness in a vacuum centrifuge (Speed Vac, Savant). This procedure is a modification of the method described previously for the rapid isolation of peptides from a number of tissues, including brain (7–9), without incurring significant losses or alterations of peptides through proteolysis (10, 11).

**Analytical and Preparative Procedures.** Identification and purification of cerebellum-specific peptides were carried out by HPLC, using post-column fluorescamine derivatization as a means of monitoring the elution of peptides. The assembly and basic configuration of this equipment was as described (12, 13). The precise details of the columns and solvent systems used are detailed in the appropriate figure legends.

Amino acid analysis was carried out on 200 pmol of peptide after hydrolysis in 6 M HCl at 110°C for 24 hr (14, 15). Approximately 500 pmol of each peptide was subjected to microsequencing by using an Applied Biophysics model 470A Sequencer. Amino acids released from each cycle were identified by HPLC on an ultrasphere ODS column (Beckman) using a trifluoroacetic acid/acetonitrile buffer system (16–18). The sequence of the carboxyl terminus was determined by a timed carboxypeptidase Y digestion of ≈160 pmol of peptide, according to the technique of Jones *et al* (19).

**Synthesis of Peptides and Confirmation of Sequence.** Peptides were synthesized having the primary amino acid sequences derived for the cerebellum-specific peptides using solid-phase techniques (20). The purified synthetic peptides were then used for chromatographic comparison with the native peptides from cerebellum using ion-pairing HPLC.

**Preparation of Cerebellar Synaptosomes.** Synaptosomes were prepared from 6-week-old rat cerebellum by the technique of Hajos *et al.* (21), as modified by Rochel *et al.* (22). The P0, P1, and P2 pellets were immediately solubilized in 6 M guanidine hydrochloride and processed as for brain samples. Sufficient guanidine hydrochloride was added to the S2 supernatant to bring it to 6 M with respect to guanidine and was then handled as described above. The presence of sucrose did not affect the recovery of the cerebellar peptides

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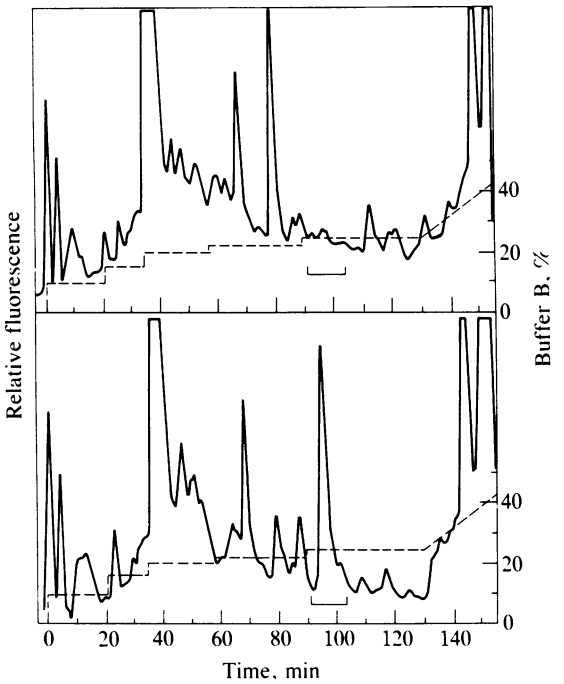


FIG. 1. HPLC analysis of peptides from cerebellum (*Lower*) and from brain minus cerebellum (*Upper*). Peptides isolated from 7 g of tissue were resuspended in 1 ml of 0.1% trifluoroacetic acid in water and injected onto a 0.46 × 25 cm Altex ODS column. The column was pre-equilibrated in 0.1% aqueous trifluoroacetic acid and was eluted at a flow rate of 1 ml/min in an increasing gradient (dashed line) of acetonitrile in 0.1% trifluoroacetic acid. Peptides were detected in the effluent by the fluorescamine derivatization procedure (12, 13). Bracketed regions indicate the fractions that were pooled for further analysis and purification.

Table 1. Amino acid analysis of the cerebellins

| Amino acid | Cerebellin* | | des-Ser[1]-cerebellin† | |
|---|---|---|---|---|
| | Mol of amino acid/ mol of peptide | Residues/ mol of peptide | Mol of amino acid/ mol of peptide | Residues/ mol of peptide |
| Asx | 0.9 | 1 | 0.9 | 1 |
| Thr | 0.9 | 1 | 0.9 | 1 |
| Ser | 3.7 | 4 | 2.8 | 3 |
| Glx | 0 | 0 | 0 | 0 |
| Pro | 0 | 0 | 0 | 0 |
| Gly | 0.9 | 1 | 0.9 | 1 |
| Ala | 3.0 | 3 | 3.1 | 3 |
| Val | 1.0 | 1 | 1.0 | 1 |
| Met | 0 | 0 | 0 | 0 |
| Ile | 1.0 | 1 | 1.0 | 1 |
| Leu | 0 | 0 | 0 | 0 |
| Tyr | 0 | 0 | 0 | 0 |
| Phe | 1.0 | 1 | 1.0 | 1 |
| His | 1.0 | 1 | 1.0 | 1 |
| Lys | 1.0 | 1 | 1.0 | 1 |
| Arg | 1.0 | 1 | 1.0 | 1 |
| Cys | 0 | 0 | 0 | 0 |
| Trp | 0 | 0 | 0 | 0 |

After sequential purification on reversed-phase (Fig. 1) and ion-pairing (Fig. 2) HPLC, 200-pmol aliquots of each fluorescamine-positive peak were taken for amino acid analysis (14, 15). The determined yields of the two peptides were as follows: early eluting peak (cerebellin), 19 pmol per cerebellum (85 pmol/g wet weight); late eluting peak (des-Ser[1]-cerebellin), 29 pmol per cerebellum (130 pmol/g wet weight).

*$M_r$, 2048.

†$M_r$, 1943.

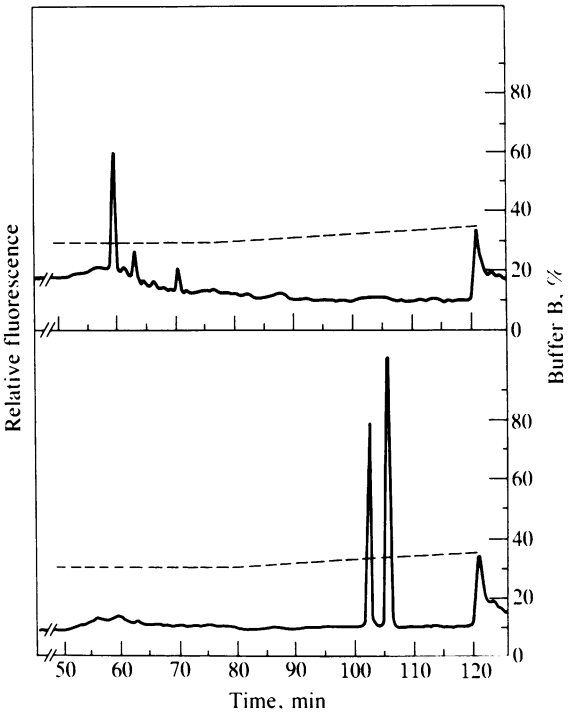


FIG. 2. Ion-pairing HPLC of cerebellum-specific peptides. (*Upper*) Brain minus cerebellum; (*Lower*) cerebellum. The indicated fractions were dried after elution from the reversed-phase column cited in Fig. 1 and resuspended in 1 ml of 0.1% aqueous trifluoroacetic acid containing 0.14% (wt/vol) octyl sodium sulfate (Eastman). The samples were injected onto an ion-pairing HPLC column (Altex IP; 0.46 × 25 cm) previously equilibrated in resuspension buffer. The column was eluted at a flow rate of 1 ml/min with an increasing gradient of acetonitrile (dashed line) in 0.1% aqueous trifluoroacetic acid containing 0.14% (wt/vol) octyl sodium sulfate. Detection was by fluorescamine derivatization (12, 13).

(data not presented). Parallel samples were analyzed for protein (23).

## RESULTS

To identify cerebellum-specific peptides, guanidine hydrochloride extracts of cerebellum and residual brain regions were desalted on Waters Associates $C_{18}$ Sep-Paks and subjected to HPLC analysis. By initially comparing elution profiles of peptides from cerebellum and from brain minus cerebellum, a fluorescamine-positive peak was identified that was apparently unique to the cerebellum. Elution conditions were optimized for the resolution of this region of the chromatogram (Fig. 1) to permit further purification. Fractions from the reversed-phase HPLC column containing the cerebellum-specific material were pooled as indicated in Fig. 1, as were the equivalent fractions from a brain minus cerebellum sample chromatographed under identical conditions. Upon further chromatography on ion-pairing HPLC using octyl sodium sulfate as pairing reagent, the material from cerebellum was resolved into two fluorescamine-positive peaks (Fig. 2). No comigrating material was observed in the brain minus cerebellum sample (Fig. 2).

Amino acid analysis of the two cerebellum-specific peaks showed them to differ only in the presence of an extra serine residue in the earlier eluting peak (Table 1). It was, therefore, inferred that the peptides were identical, save for an additional serine at either the amino or carboxyl terminus.

The purified peptides were sequenced by gas-phase Edman degradation, with the results shown in Fig. 3. The early eluting peak is a hexadecamer, which we refer to as cerebellin. The second peptide is a pentadecamer with the same pri-

Cerebellin

$NH_2$-Ser-Gly-Ser-Ala-Lys-Val-Ala-Phe-Ser-Ala-Ile-Arg-Ser-Thr-Asn-His-OH

Des-Ser$^1$-Cerebellin

$NH_2$-Gly-Ser-Ala-Lys-Val-Ala-Phe-Ser-Ala-Ile-Arg-Ser-Thr-Asn-His-OH

FIG. 3. Primary amino acid sequences of cerebellin and des-Ser$^1$-cerebellin. Approximately 500 pmol of cerebellin and des-Ser$^1$-cerebellin (early and late eluting peaks, respectively) were sequenced by gas-phase Edman degradation.

mary amino acid sequence as cerebellin, except that it lacks the amino-terminal serine (Fig. 3) and was, therefore, designated des-Ser$^1$-cerebellin. Although the derived primary structures (Fig. 3) account for all the amino acids determined after hydrolysis (Table 1), the carboxyl-terminal sequence was also confirmed by carboxypeptidase Y digestion. As predicted from sequence analysis, amino acids were released from cerebellin and des-Ser$^1$-cerebellin by carboxypeptidase Y in the order histidine, asparagine, threonine (data not presented). As a final verification of the sequence, both peptides were synthesized and subjected to ion-pairing HPLC in the presence of native cerebellar peptides (Fig. 4). Synthetic peptides cochromatographed with their natural counterparts. Neither of these amino acid sequences is present in the Dayhoff library.

To more fully establish the distribution of cerebellin and des-Ser$^1$-cerebellin, various brain regions and extraneural tissues were subjected to HPLC analysis (Fig. 5). Using synthetic cerebellin peptides as markers, it was observed that only cerebellum contained detectable levels of either peptide species (Fig. 5). When synthetic cerebellin was added to guanidine hydrochloride extracts of cortex and cerebellum and the samples were carried through the ion-pairing HPLC procedure, the overall recovery of peptides was calculated to be 65% (data not presented). Thus, it is unlikely that differences in the observed levels of cerebellin in brain tissue could be attributed to various levels of proteolysis or recovery during isolation. Further experiments have established that the cerebella but not cerebral cortices of mice, rabbits, and guinea pigs contain chromatographically homologous peptides to the cerebellins (data not presented). While the chicken cerebellum contains no cochromatographic peptides (data not presented), the cerebellins do appear to have a wide distribution amongst mammals.

When synaptosomes were prepared from cerebellum by the procedure of Hajos *et al.* (21), both cerebellin and des-Ser$^1$-cerebellin were enriched in the P2 fraction (Table 2), which contains the majority of synaptosomes in such preparations (21). These results, and other unpublished data, indicate that both peptide species are sequestered *in vivo* in a subcellular organelle that may be associated with the synapse.

## DISCUSSION

By combining a rapid peptide isolation–desalting procedure (7–11) with analytical HPLC, it has been possible to identify two peptides that are substantially more abundant in the cerebella of several mammalian species than in any other brain region analyzed. Both peptides have been isolated from rat cerebellum by sequential chromatography on reverse phase followed by ion-pairing HPLC, and their primary amino acid sequences were determined by gas-phase Edman degrada-

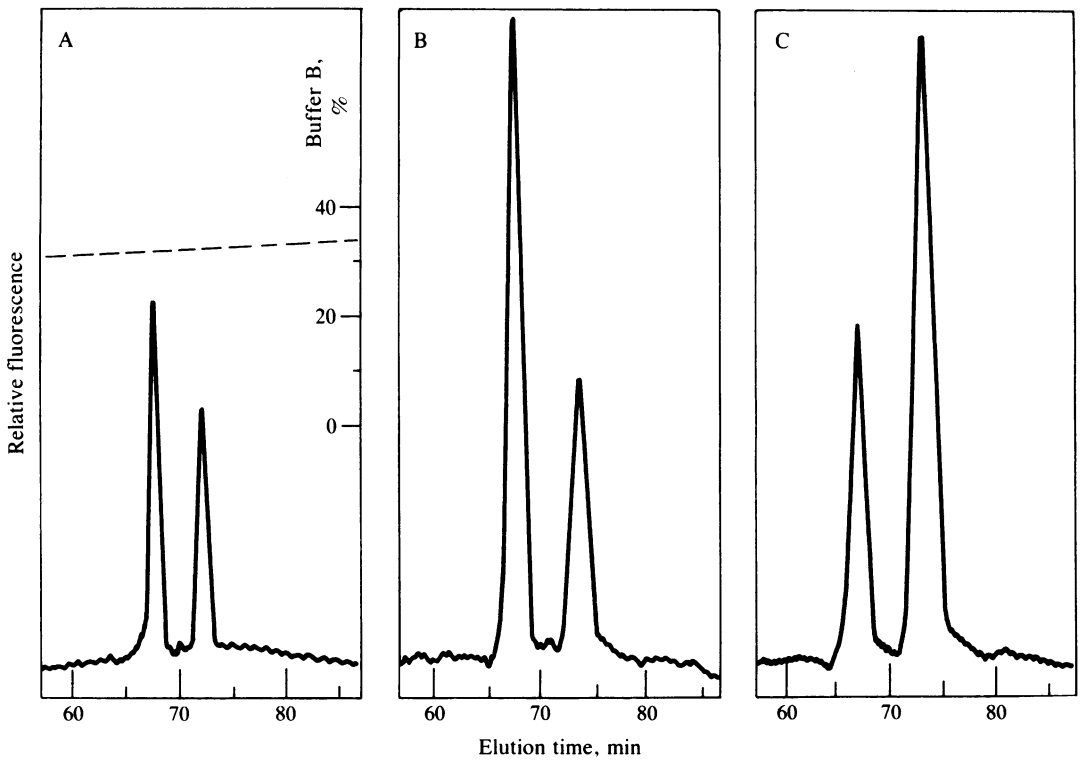


FIG. 4. Cochromatography of native and synthetic cerebellin peptides. Native cerebellum-specific peptides were chromatographed on an ion-pairing HPLC column as described in Fig. 2, either alone (*A*), in the presence of 50 pmol of synthetic cerebellin (*B*), or in the presence of 50 pmol of synthetic des-Ser$^1$-cerebellin (*C*). Details of elution and detection are as in Fig. 2.

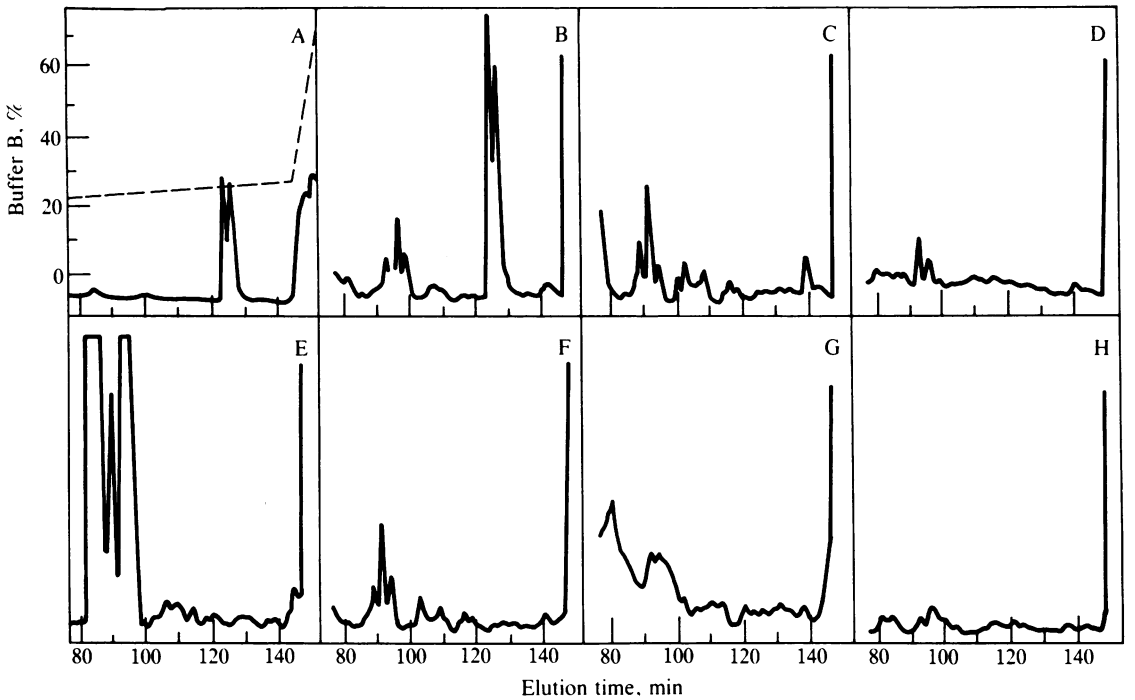


FIG. 5. Tissue distribution of cerebellin and des-Ser[1]-cerebellin. Guanidine hydrochloride extracts were made from the indicated tissues. Synthetic cerebellins (50 pmol) were used as markers. HPLC analysis was carried out on an Altex IP column (0.46 × 25 cm) pre-equilibrated in 0.1% aqueous trifluoroacetic acid containing 1.2 g/liter of hexane sulfonic acid (Eastman) as ion-pairing reagent. Elution was with an increasing gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid containing 1.2 g/liter of hexane sulfonic acid at a flow rate of 0.5 ml/min. Peptide detection was achieved by post-column fluorescamine derivatization (12, 13). (*A*) Elution of 50 pmol each of cerebellin and des-Ser[1]-cerebellin; (*B–H*) fluorescamine yield derived from 0.5 g of cerebellum (*B*), 0.5 g of brain stem (*C*), 0.25 g of spinal cord (*D*), 0.75 ml of whole blood (*E*), 0.5 g of thalamus (*F*), 0.5 g of liver (*G*), and 0.25 g of olfactory bulb (*H*).

tion and carboxypeptidase Y analysis. Sequence was finally confirmed by synthesis. The larger of the two peptides is a hexadecamer termed cerebellin with a molecular weight of 2048. The second peptide, lacking one serine residue at the amino terminus, is referred to as des-Ser[1]-cerebellin.

At this juncture, we are unable to state whether further cerebellum-specific peptides exist in sufficient numbers or in adequate concentrations to warrant their systematic study by HPLC. However, the properties of cerebellin and des-Ser[1]-cerebellin revealed in this investigation highlight a number of advantages of this proposed approach. (*i*) The identification of these peptides required neither antigenicity nor any overt biological function. (*ii*) Since the extraction procedure minimizes proteolysis, subsequent HPLC analysis is quantitative. (*iii*) The des-Ser[1]-cerebellin species was presumably derived either by processing from a larger precursor or by the action of a serine aminopeptidase on cerebellin. Both of these scenarios would require the enzymatic cleavage of a serine-glycine peptide bond (Fig. 3). Such a specific proteolytic cleavage would not have been predicted from our current knowledge of protein processing. Thus, we may conclude that not only must such processing enzymes exist but also that the existence of the des-Ser[1]-cerebellin peptide could not have been predicted from the primary structure of the precursor if it were known. Furthermore, in those instances in which cell-specific markers have been identified by gel electrophoresis (24) or from cDNA libraries (25), these results show that the presence of specific peptide fragments could not be inferred from primary sequence. (*iv*) Based on the primary amino acid sequence information, synthetic peptides have been made. By coupling the latter to carrier proteins, sequence-directed antisera have been raised that have been used to demonstrate the localization of cerebellin in the Purkinje cells of rat cerebellum (unpublished

Table 2. Subcellular distribution of the cerebellins

| | Cerebellin content | | des-Ser[1]-cerebellin content | |
|---|---|---|---|---|
| | pmol per mg of protein | % total input | pmol per mg of protein | % total input |
| Intact cerebellum | 3.12 | 100 | 2.98 | 100 |
| P0 (1000 × *g*; 1 min) | 2.25 | 21 | 3.16 | 31 |
| P1 (1000 × *g*; 10 min) | 3.35 | 16 | 3.40 | 17 |
| P2 (14,500 × *g*; 20 min) | 11.22 | 35 | 9.16 | 30 |
| S2 (14,500 × *g* supernate) | 0 | 0 | 0 | 0 |

Cerebella from eight rats were subfractionated by the procedure of Hajos *et al.* (21) as modified by Rochel *et al.* (22). The cerebellin and des-Ser[1]-cerebellin content of each subcellular fraction was determined by ion-pairing HPLC, using octyl sodium sulfate as pairing reagent as described in Fig. 2. Areas under the fluorometer traces were integrated for the two peptides, and this was quantitated against a calibration curve with synthetic peptides. Protein was determined on parallel samples. The reported data are the mean values derived from three separate experiments with an interexperimental variation of <20%. Also shown is the recovery of each peptide in the various fractions as a percentage of the peptide present in an equivalent amount of intact cerebellum used for the fractionation.

data). Thus, when dealing with small peptides, it is possible to obtain the kinds of cell-specific information available with the more conventional immunological approaches to the identification of marker molecules. (*v*) While the fundamental reason for embarking on this study was to identify markers for neurodevelopmental investigations, it should be remembered that peptides represent potential regulatory molecules. Since the cerebellum is a poor source of many of the recognized neuromodulatory peptides (26, 27), the identification of a cerebellum-specific species is the more relevant. This is further emphasized by the observation that both cerebellin and des-Ser$^1$-cerebellin are enriched in the P2 synaptosomal fraction of cerebellum. (*vi*) Finally, it would be anticipated that the production of peptides, particularly those involved in neuromodulation, would be a late-developing characteristic of neurons. This is indeed the case for cerebellin production in cerebellar Purkinje cells (unpublished data). Thus, these peptides are significant probes with which to investigate the maturation of the rodent cerebellum.


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