Journal of Peptide Science
J. Peptide Sci. **4**: 211–225 (1998)

Peptides from Bovine Brain: Structure and Biological Role

ANDREI A. KARELIN, MARINA M. PHILIPPOVA, ELENA V. KARELINA, BORIS N. STRIZHKOV, GALINA A. GRISHINA, IGOR V. NAZIMOV and VADIM T. IVANOV*

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

Received 22 May 1997
Accepted 24 July 1997

Abstract: Fractionation of bovine brain extracts followed by automatic Edman sequencing of individual components resulted in identification of 107 endogenous peptides formed from functional proteins (haemoglobin, myelin basic protein, cytochrome *c* oxidase, etc) or unknown precursors. Several of the newly identified brain peptides demonstrate different types of biological activity; some of the substances show considerable overlap with the known biologically active peptides. It is suggested that these peptides should participate in regulation of extracellular and intracellular biochemical processes. A concept of 'tissue-specific peptide pool' is formulated describing a novel system of peptidergic regulation, complementary to the conventional hormonal and neuromodulatory systems. According to that description functional proteins provide their proteolytically derived fragments for maintaining the tissue homeostasis by modulating the availability of peptide receptors to respective 'true' ligands. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Endogenous peptides; proteolytic degradation; functional protein; biologically active peptides

INTRODUCTION

Isolation and structure–function studies of novel active peptides from different tissue extracts usually follow the standard 'from function to structure' pathway. This approach implies consecutive multistage separation of the initial mixture complemented by systematic control of biological activity of isolated fractions in an appropriate screening test-system, adequately reflecting the biological function of interest. The result of such a study is isolation and identification of a homogeneous substance exhibiting the given activity. After more detailed study the discovered substance usually reveals several other biological activities (for example see [1]). The reverse scheme, 'from structure to function' leads to isolation and structural elucidation of a maximum set of homogeneous substances from the initial material. Such an approach provides a starting point for the synthesis and study of biological activity of a wide range of compounds [2, 3]. Immunoscreening using antibodies to active substances with the established structure and function parameters combines features of both strategies [4–6].

The above approaches resulted in the isolation of more than 1000 naturally occurring peptides, for which a wide spectrum of biological activities has been demonstrated or postulated. In general, the isolated peptides might be divided into two groups. The first group includes the peptides cleaved from specific and, presumably inactive precursors, proteins from which biologically active peptides are released by specific proteolysis usually performed by enzymes with trypsin-like specificity [7–9]. Such peptides are secreted from the cells, which is also a highly specific, often Ca^{2+}-dependent process [10]. These are 'classical' peptide bioregulators to which belong most of the hormones, parahormones and neurotransmitters [1, 7–13]. The second group includes peptides cleaved from the proteins possessing their own functions in the organism. Peptides of that group have so far been studied in less detail; however, their number has dramatically increased in the past few years. These peptides were shown to originate from cytochrome *c* oxidase [14], myelin basic protein [2, 15], γ-globulins [4, 16], serum

*Address for correspondence: Vadim T. Ivanov, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russia.

CCC 1075-2617/98/030211–15

albumin [17] and some other proteins [6, 18, 19], the main source of such peptides being a haemoglobin ([20–29]; for review see [3]).

The present work follows the 'from structure to function' approach and deals with the analysis of bovine brain extracts for individual peptide components. As a result several dozens of new endogenous peptides have been discovered, the majority of them being proteolytic fragments of well-known functional proteins.

MATERIALS AND METHODS

Preparation of Total Bovine Brain, Cortex and Subcortex Structures of Bovine Brain

Bovine brain tissue (2 kg) was stored in liquid nitrogen. Brain portions of 100 g were homogenized in a knife homogenizer in 50 ml of 1 M acetic acid. The obtained mixture was centrifuged at 10,000 rev/min. The supernatant was lyophilized. All procedures were carried out within 20–30 min at 0°C. Some 100 g of lyophilizate were obtained from 2000 g of brain tissue.

The cortex was separated from the rest of the brain (subcortex) layer by layer as soon as the unfreezing process at 0°C allows. The extracts were prepared as described above.

Size-exclusion Chromatography

The size-exclusion fractionation was performed using a liquid chromatography set (Pharmacia/LKB, Sweden) equipped with a Sephadex G-25 sf column (2.5 × 85 cm) equilibrated with 0.1 M acetic acid. Some 400 mg of lyophilized preparation were dissolved in 0.1 M acetic acid and subjected to

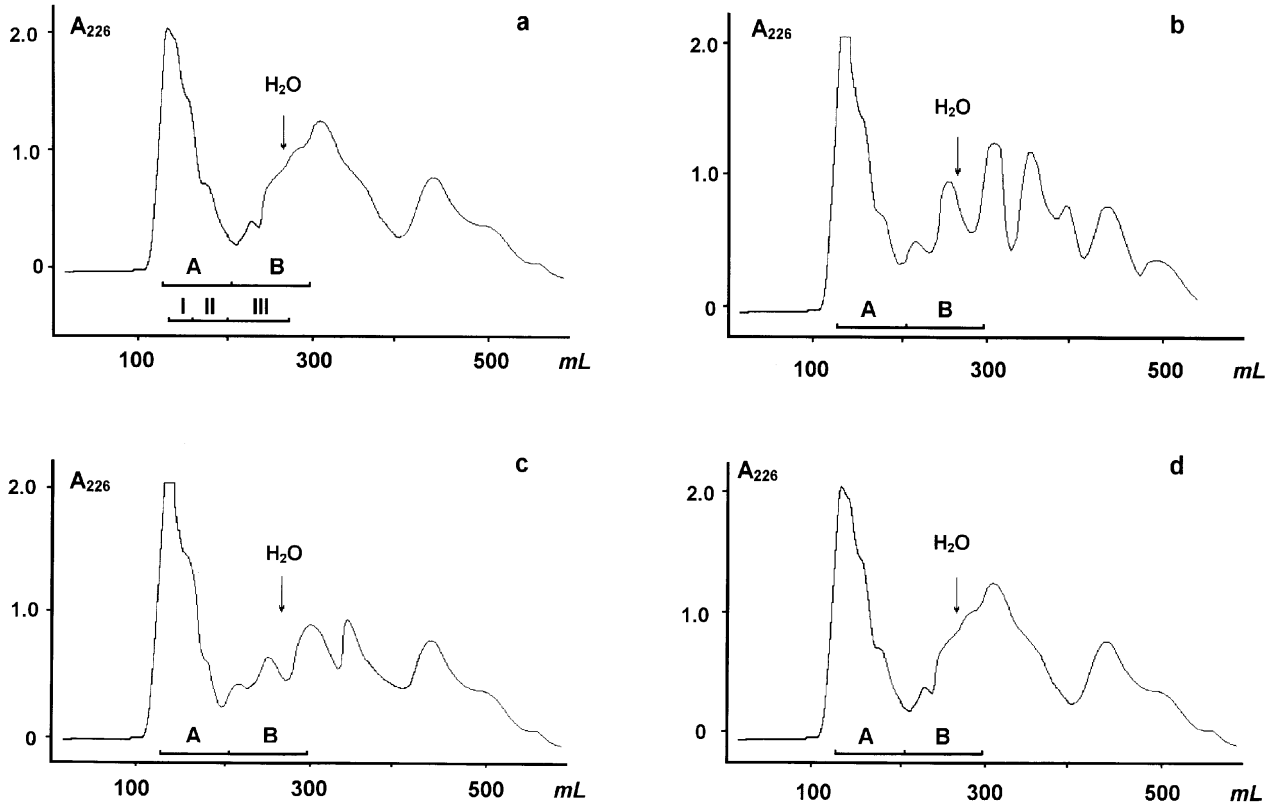

Figure 1 Size-exclusion chromatography of acidic extract of bovine brain (200 mg) preparations on Sephadex G-25 sf column (2.5 × 85 cm), equilibrated with 0.1 M acetic acid. Separation conditions are marked. (a) Preparations of total bovine brain extracts; (b) preparation of subcortex structures; (c) preparation of cortex; (d) brain preparation obtained after 6 h of incubation at +10 °C.

separation under conditions described in the legend to Figure 1. The obtained fractions were lyophilized.

Reverse-phase HPLC

Analytical separation of fractions obtained after size-exclusion chromatography was performed using Nucleosil C_8 120/7 μ (4.6 × 250 mm) equilibrated in 0.1% trifluoroacetic acid. The samples were eluted by linear gradient of acetronitril in 0.1% trifluoroacetic acid (buffer A, 0.1% TFA water solution; buffer B, 0.1% TFA in 80% acetonitril). Separation conditions are described in the legends to Figures 2 and 3.

Semi-preparative separation of fractions obtained after size-exclusion chromatography was performed using Nucleosil C_8 120/7μ (10 × 250 mm) equilibrated in 0.1% trifluoroacetic acid (buffer A, 0.1% TFA water solution; buffer B, 0.1% TFA in 80% acetonitril). The samples were eluted by the linear gradient of acetronitril in 0.1% trifluoroacetic acid Separation conditions are described in the legends to Figures 4–6. The peaks corresponding to the main substances were collected, lyophilized and subjected to separation on Nucleosil 120/5μ C_{18} cartridge (4.0 × 250 mm) in the linear acetonitril gradient from 8 to 40%. The elution profiles were obtained using an integrator (Ampersend, Russia) at 226 nm. The optical density values given in Figures 2–6 correspond to the ratio 2.56 OD = 1800 mV. Analysis of the elution profiles was performed by Multi-Chrom V2.60 program (Ampersend, Russia).

Reproducibility of the data presented in this paper was checked by comparing the elution profiles in three independent experiments. In several cases

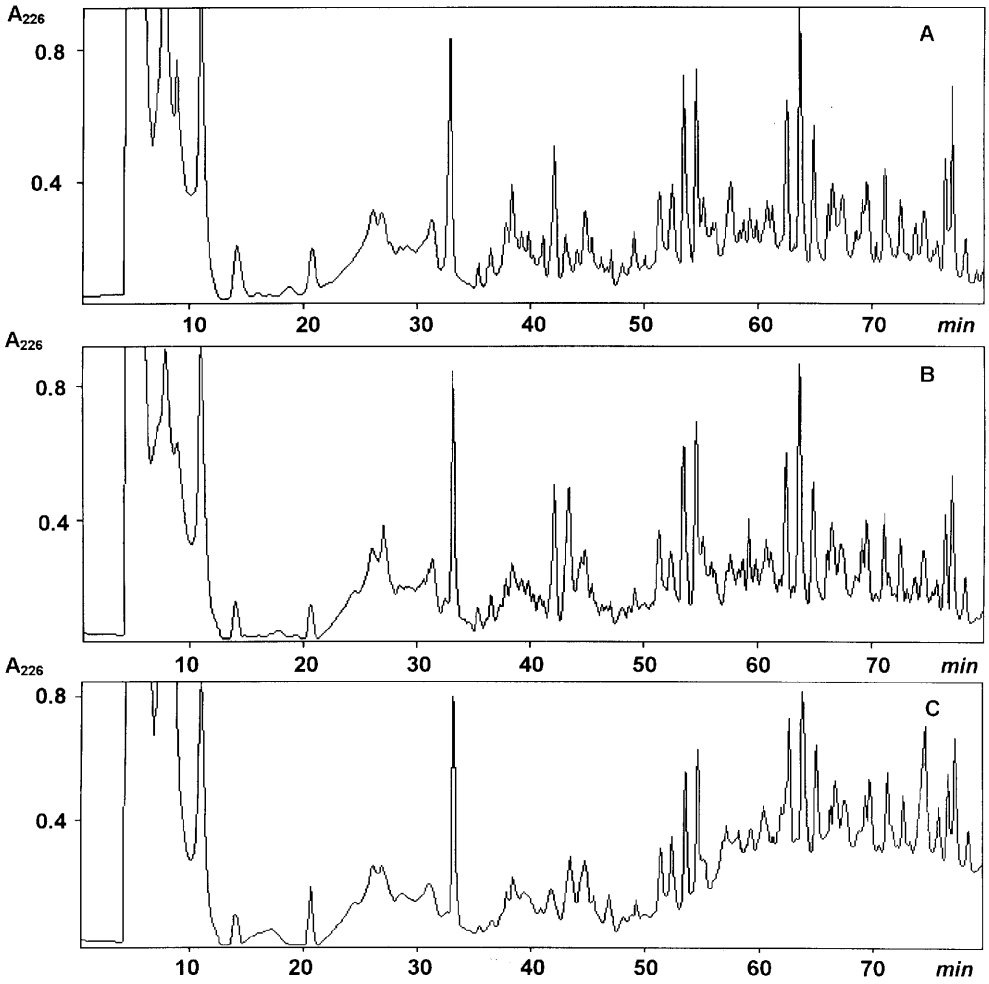

Figure 2 RP-HPLC of fractions B (Figure 1 (a)) on Nucleosil 7μC_8 (4.6 × 250 mm) column equilibrated with 0.1% TFA. Elution was performed at the following conditions: isocratic elution in initial buffer A, 0–20 min; gradient from 0 to 60% of buffer B, 20–80 min; flow rate, 1 ml/min; wavelength detection, 226 nm; sample size, 0.4 mg. (**A**) and (**B**) independent batches of total bovine brain preparation; (**C**) total bovine brain preparation obtained in modified conditions (Figure 1 (d)).

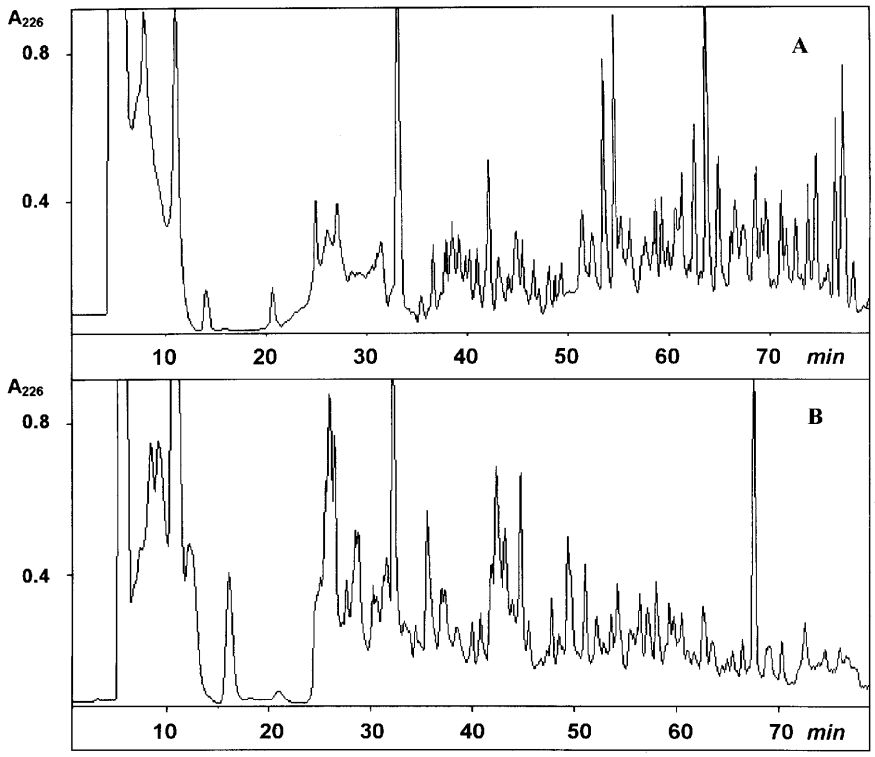

Figure 3 RP-HPLC of fractions B of the (**A**) subcortex and (**B**) cortex extracts (Figure 1 (b) and (c)) on Nucleosil 7μC_8 (4.6 × 250 mm) column equilibrated with 0.1% TFA. Elution was performed at the following conditions: isocratic elution in initial buffer A, 0–20 min; gradient from 0 to 60% of buffer B, 20–80 min; flow rate, 1 ml/min; wavelength detection, 226 nm; sample size, 0.4 mg.

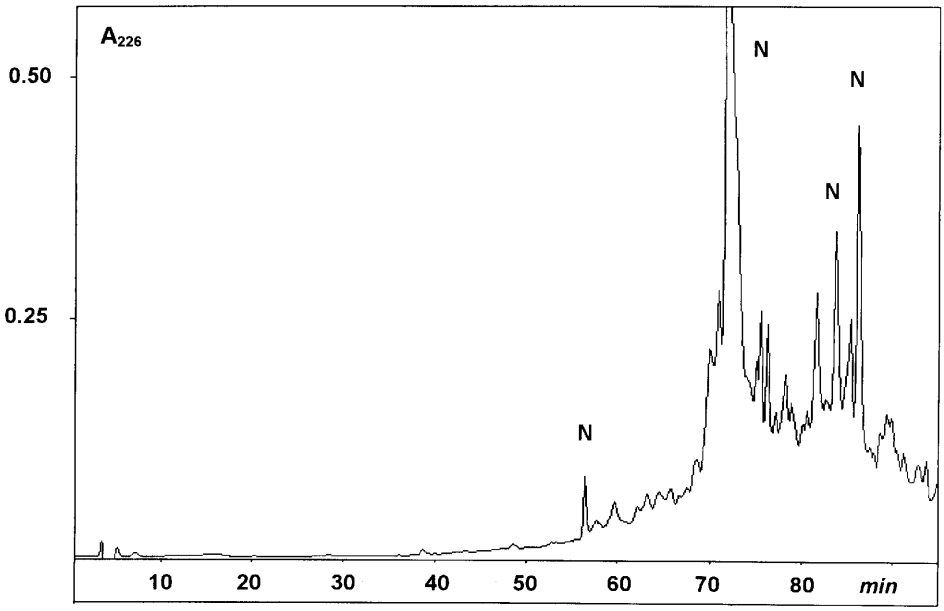

Figure 4 RP-HPLC of fraction I (Figure 1 (a)) on Nucleosil 7μC_8 (10 × 250 mm) column equilibrated with 0.1% TFA. Elution was performed by acetonitril concentration gradient (from 0 to 60% of buffer B, 0–80 min; flow rate, 2.5 ml/min); wavelength detection, 226 nm; sample size, 1.0 mg. N, peaks corresponding to substances for which the amino acid sequence could not be determined.

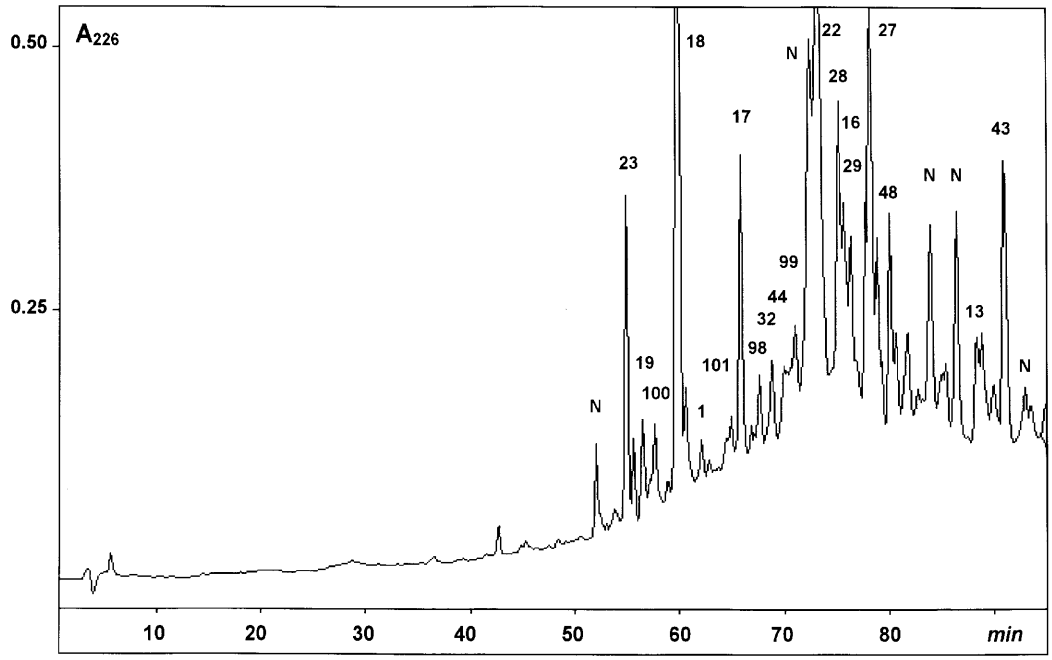

Figure 5 RP-HPLC of fraction II (Figure 1 (a)) on Nucleosil 7μC_8 (10 × 250 mm) column equilibrated with 0.1% TFA. Elution was performed by acetonitril concentration gradient (from 0 to 50% of buffer B, 0–80 min; flow rate, 2.5 ml/min); wavelength detection, 226 nm; sample size, 1.3 mg. The fractions containing the identified peptides are marked by numbers. The numbers correspond to sequences represented in Tables 2–4. N, peaks corresponding to substances for which the amino acid sequence could not be determined.

respective sequencing analysis was used to verify the reproducibility of the results.

Peptide Sequencing

Amino acid sequences were determined by means of gas-phase sequencer (Model 477A, Applied Biosystems). C-terminal amino acid residues of the obtained peptides were established according to the data of two or three repeated sequencing procedures.

RESULTS AND DISCUSSION

Isolation and Identification of Peptide Components from Bovine Brain

Total bovine brain extract was chosen as the principal source of peptides subjected to structural study. The cortex and subcortical brain structures were used as auxiliary sources. All tissues were treated by a standard procedure under conditions ensuring minimal loss of original peptides. For comparison, one experiment was carried out with the brain sample left for 6 h at 10 °C before extraction. The lyophilizates were fractionated on a Sephadex G-25 sf column initially giving rise to fractions A (2.0–4.0 kDa) and B (0.3–2.0 kDa) (Figure 1 (a)–(c)). All three tissues gave highly reproducible elution profiles.

The yield of the material in fractions A and B of the total brain, cortex and subcortex is given in Table 1. Fractions A and B were subjected to further separation on Nucleosil C_8 column giving rise to quite complex profiles and numerous subfractions. As with size-exclusion chromatography, the RP-HPLC of fraction B showed excellent reproducibility for total brain extracts (Figure 2 (a) and (b)).

Storage of the brain sample resulted in noteable changes both in the size-exclusion (Figure 1 (d)) and

Table 1 The Yield of the Material in the Fractions of Tissue Extracts (mg per g of tissue)

	Fraction				
Tissue	A	B	I	II	III
Total brain	2.3	4.2	1.9	0.8	3.7
Cortex	1.2	3.6	–	–	–
Subcortex	3.5	6.2	–	–	–

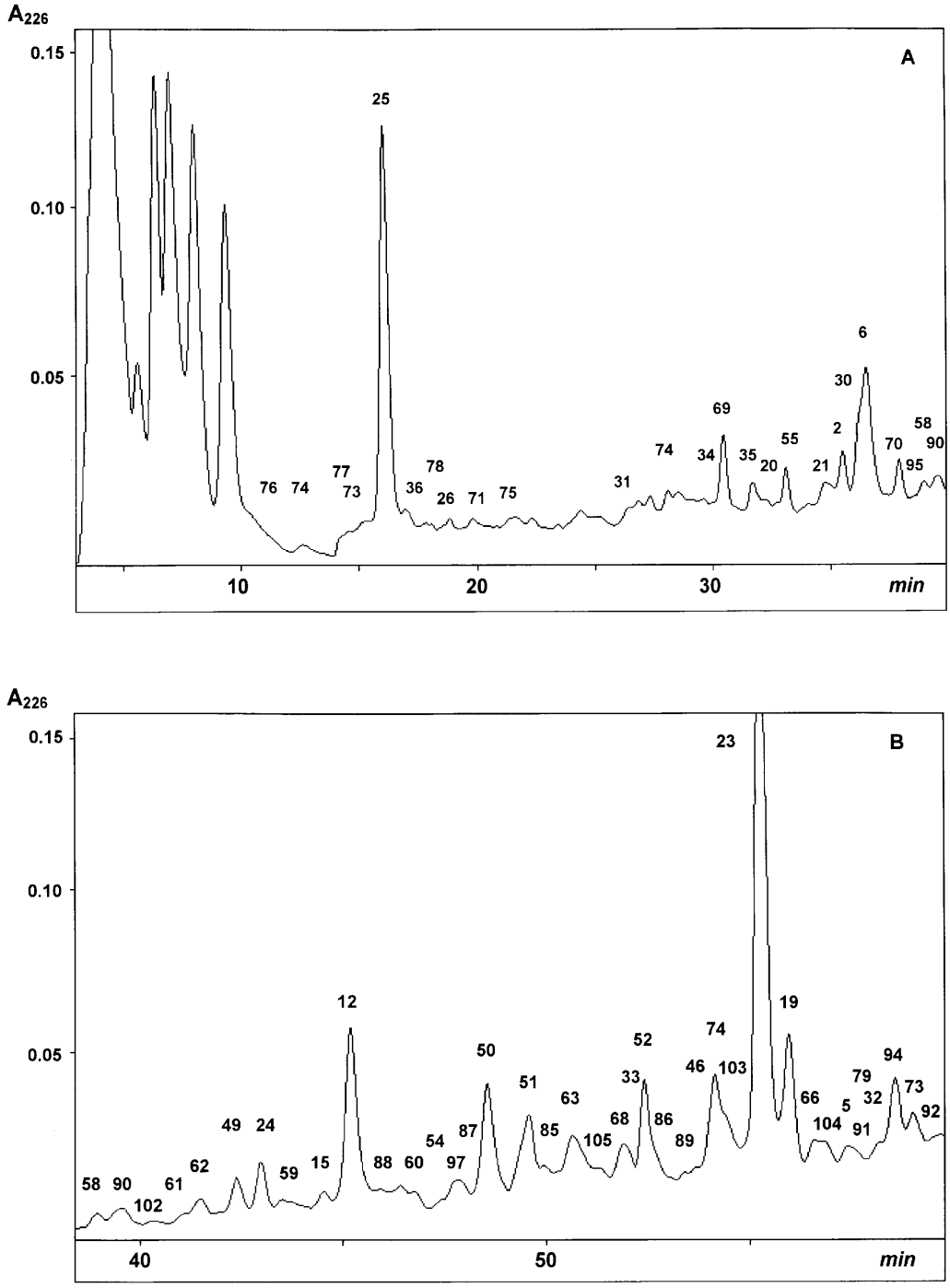

Figure 6 RP-HPLC of fraction III (Figure 1 (a)) on Nucleosil 7μC_8 (10 × 250 mm) column equilibrated with 0.1% TFA. Elution was performed by acetonitril concentration gradient (from 0 to 60% of buffer B, 0–80 min; flow rate, 2.5 ml/min); wavelength detection, 226 nm; sample size, 1.8 mg. The peaks and zones containing the identified peptides are marked by numbers. The numbers correspond to sequences represented in Tables 2–4. N, peaks corresponding to substances for which the amino acid sequence could not be determined. (**A**), (**B**) and (**C**) consecutive parts of the elution profile.

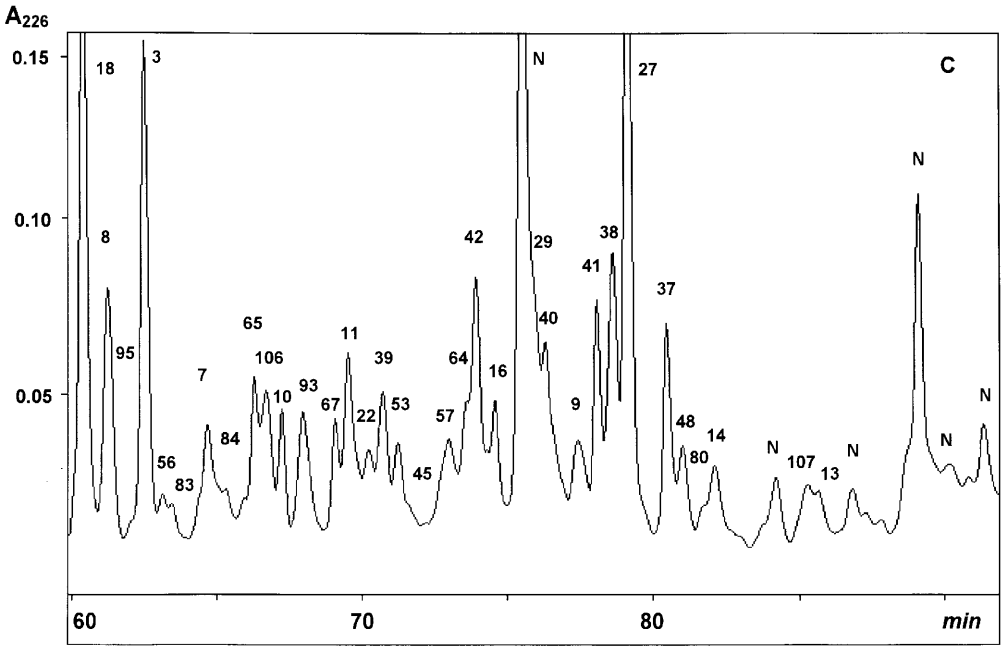

Figure 6 (*continued*)

RP-HPLC (Figure 2 (c)) profiles. However, as seen from the later figure all major peaks and most of the minor ones remain in place in the low molecular fraction even under these conditions. That result allows us to conclude that components of the studied extract reflect in principle the endogenous *in vivo* composition of the bovine brain.

A comparison of low molecular profiles from the total brain (Figure 2 (a) and (b)) and substructures (Figure 3) points to several components present in one subfraction and missing in the other. All these components are present in the total brain extract. In other words, the peptide composition is tissue-specific.

Similar attempts to characterize the composition of the high molecular fraction A were unsuccessful owing to unsatisfactory resolution. Therefore in further experiments we added one more subfraction in the size-exclusion chromatographic scheme as shown in Figure 1 (a). The new scheme led to fractions I, II and III, each of which was successfully separated into individual components (Figure 4–6). The overall number of major components (i.e. components present at 50 pmol/g of tissue or higher) is not less than 200. These components were rechromatographed on the Nucleosil 5μC_{18} column and subjected to gas phase automatic sequencing. As a result, complete or partial amino acid sequences of 107 peptides were established. According to our estimate, about 80% of peptides from fractions II and III (containing respectively peptides 20–40 and 3–20 amino acid residues in length) present at >0.1 nmol/g tissue levels were sequenced.

Attempts to determine amino acid sequences of the high molecular fraction I were not successful. Further studies employing methods of protein chemistry (as demonstrated by Slemmon and Flood [30]) are required in that case. As shown in Figures 5 and 6 several peptides from fractions II and III also could not be sequenced under the standard conditions (blocked N-termini ?); *ca* 20 fractions did not contain peptide material.

The amino acid sequences obtained were compared with the sequences in the PIR data bank by the original FASTA program [2]. Several completely or partially overlapping sequences were found within the known proteins which allowed to assign these peptides to their respective precursors (Tables 2 and 3). The rest of the peptides were either too short to allow assignment of the precursor or has original amino acid sequences (Table 4).

The data given in Tables 2–4 require further comment regarding the origin and biological role of discovered peptides. As already mentioned, the reproducibility and tissue specificity of peptide composition allow us to postulate the endogenous nature of the identified peptides. Another, though also indirect, argument is the fact that we were able to isolate very unstable fragments (Table 2, peptides

Table 2 Fragments of Functional Proteins Identified in Bovine Brain Extracts

No.	Amino acid sequence	Protein precursor and the postion of the identified fragment		Content (nmol/gram tissue)
Neurospecific proteins				
1	AAAAKIQASFRGHMARKKIKSGERGRKGPGPGGPGGAGR	Neurogranin (p17)	24–62	< 0.1
2	KYLASASTMDHARHG	Myelin basic protein	11–25	< 0.1
3	FGSDRGAPKRGSGK	Myelin basic protein	43–56	1.0–3.0
4	FGSDRGAPK	Myelin basic protein	43–51	0.1–1.0
5	ELEKAVVALI	S-100 B	2–10	< 0.1
Other functional proteins				
6	NKVP	Cytochrome *c* oxidase VIIA	12–15	0.1–1.0
7	IEWNPSPVGRVTPKEWREQ	Cytochrome *c* oxidase VIIB	70–88	< 0.1
8	IEWNPS	Cytochrome *c* oxidase VIIB	70–75	< 0.1
9	SHYEEGPGKNIPFSVENKWRL	Cytochrome *c* oxidase VIIIA	1–21	0.1–1.0
10	FGSGFAAPF	Cytochrome *c* oxidase VIIIA	29–37	< 0.1
11	LLPSGWVLSHMENYKKR	Cytochrome *c* oxidase IX	52–68	0.1–1.0
12	SSGAHGEEGSARMWKA	Cytochrome *c* oxidase SSG	1–16	< 0.1
13	MLQSLIKKVWIPMKPYYTQAY	6.8 K proteolipid	1–21	0.1–1.0
14	SLIKKVWIPMKPYYT	6.8 K proteolipid	4–18	< 0.1
15	FIVH	GTP-ase activator	304–307	0.1–1.0
16	MQIFVKTLTGKTITL	Ubiquitin	1–15	0.1–1.0
Haemoglobin				
17	VLSAADKGNVKAAWGKVGGHAAEYGAEALERM	α-globin	1–32	0.1–1.0
18	VLSAADKGNVKAAWGKVGGHAAEYGAEALE	α-globin	1–30	1.0–3.0
19	VLSAADKGNVKAAWGKVGGHAAEY	α-globin	1–24	1.0–3.0
20	DKGNV	α-globin	6–10	0.1–1.0
21	LSHSL	α-globin	101–105	0.1–1.0
22	ASHLPSDFTPAVHASLDKFLANV	α-globin	110–132	1.0–3.0
23	ASHLPSDFTPAVHASLDK	α-globin	110–127	0.1–1.0
24	FLANVSTVL	α-globin	128–136	0.1–1.0
25	TSKYR	α-globin	137–141	0.1–1.0
26	TSKY	α-globin	137–140	0.1–1.0
27	MLTAEEKAAVTAFWGKVKVDEVGGEALGR	β-globin	1–29	0.1–1.0
28	MLTAEEKAAVTAFWGKVKVDEVGGEALG	β-globin	1–28	0.1–1.0
29	MLTAEEKAAVTAF	β-globin	1–13	0.1–1.0
30	MLTAEEKA	β-globin	1–8	0.1–1.0
31	MLT	β-globin	1–3	0.1–1.0
32	WGKVKVDEVGGEA	β-globin	14–26	0.1–1.0
33	WGKVKVDEVG	β-globin	14–23	0.1–1.0
34	EVGGEALG	β-globin	21–28	0.1–1.0
35	EVGGEAL	β-globin	21–27	< 0.1
36	GGE	β-globin	23–25	0.1–1.0
37	LVVYPWTQRF	β-globin	31–40	1.0–3.0
38	LVVYPWTQ	β-globin	31–38	1.0–3.0
39	LVVYPWT	β-globin	31–27	0.1–1.0
40	LVVYP	β-globin	31–35	0.1–1.0
41	VVYPWTQRF	β-globin	32–40	1.0–3.0
42	VVYPWTQ	β-globin	32–38	0.1–1.0
43	VVVLARNFGGEFTPVLQADFQKVVAGVAN-?	β-globin	110–138?	0.1–1.0
44	VVVLARNFGGEFTPVLQ	β-globin	110–128	0.1–1.0
45	VVVL	β-globin	110–113	0.1–1.0
46	ARNFGGEFTPVLQ	β-globin	114–126	0.1–1.0
47	VLS	β-globin	124–126	0.1–1.0
48	FQKVVAGVANALAHRYH	β-globin	129–145	0.1–1.0

Table 3 Peptides from Bovine Brain Extracts Homologous to Protein Fragments of Cattle or Other Species

No.	Amino acid sequence	Protein	Fragment	Source	Homology (%)	Content (nmol/gram tissue)
49	FLPGH	Aspartate synthetase	180–184	Human	100	0.1–1.0
50	LNETGDEPFQYKN	Glutamate-ammonium ligase	361–373	Human	100	0.1–1.0
51	LNETGDEPFQYK	Glutamate-ammonium ligase	361–372	Human	100	0.1–1.0
52	LNETGDEPFQ	Glutamate-ammonium ligase	361–370	Human	100	0.1–1.0
53	ISWYDNEFGYSNRV	Glyceraldehyde-phosphate dehydrogenase	335–349	Human	100	0.1–1.0
54	LMYP	Procollagenase	233–236	Human	100	0.1–1.0
55	EGEPNL	Mouse secretory component	440–446	Mouse	100	0.1–1.0
56	PLFP	Acetylcholine receptor	11–14	Pig	100	0.1–1.0
57	TFSYGRALQA	Fructoso-diphosphate aldolase A	299–308	Mouse	100	<0.1
58	FISNHAY	Fructoso-diphosphate aldolase A	358–366	Mouse	100	0.1–1.0
59	SRDKR-NH_2	Immunoglobulin (ε-chain)	337–341	Mouse	100	<0.1
60	FPNEPM	Cytochrome P450	401–406	Mouse	100	0.1–1.0
61	VVGQV	Keratin 47K, type I	121–125	Mouse	100	0.1–1.0
62	VLGQV	Complement C5 factor	1049–1053	Human	100	0.1–1.0
63	YAYYY	Multidrug resistance protein	113–117	Mouse	100	<0.1
64	EEGPGKNIPFSVENKQR	Cytochrome *c* oxidase VIIIA	4–20	Cattle	95	0.1–1.0
65	INLFFIVL	Na-channel protein	1576–1582	Rat	75	<0.1
66	TQLPAEEI	Protachikinin	15–22	Cattle	85	<0.1
67	IQVFAEPKVLYVTRL	Neuron adhesion factor	562–576	Cattle	30	<0.1
68	INNPFIL	LANT-6 peptide	1–6	Chicken	65	<0.1
69	YEQLSGK	Cytochrome *c* oxidase VIIIA	3–9	Cattle	55	0.1–1.0

3 and 4) of the myelin basic protein which spontaneously cleave its Asp-Arg bond upon storage even at 65 °C [2]. Finally, haemoglobin fragments similar or very close to those represented in Table 2 were isolated earlier from human pituitary gland material [20], cerebellum [21], liquor [22] and gingival crevicular fluid [23]; pig hypothalmus [24] and bone marrow [25]; bovine brain [26–28] and bone marrow [29].

The peptides listed in Tables 2–3 apparently result from tissue-specific proteolysis of functional precursor proteins. The latter include both, intracellular proteins (such enzymes as cytochrome *c* oxidase, glutamate-ammonium ligase, etc.) and extracellular proteins (immunoglobulins, complement factor, etc.) as well as membrane-associated proteins (acetylcholine receptor, sodium channel protein, etc.). In some cases fragments are found of the protein present in very low levels, e.g. protakhikinin. At the same time fragments of such highly represented proteins as the intracellular cytochrome *c* or the extracellular serum albumin were not found in the studied extracts.

The origin of haemoglobin fragments deserves special attention. Earlier we demonstrated that relatively long fragments of *ca.* 30 amino acid residues are found within erythrocytes [31] while shorter fragments containing 5–17 residues are released from red blood cells [3]. Therefore we assume that longer fragments in Table 2, such as 17, 18, 27, 28 and 43, reflect the residual erythrocyte content in brain blood vessels. Among the shorter fragments some represent the peptides released from erythrocytes and the major part consist of their further degradation products during the tissue-specific proteolysis [3].

Biological Properties of Identified Peptides

Recently neokyotorphin and its fragment (1–4) (peptides 25 and 26 in Table 2) were identified in the brain of ground squirrels [32] as well as in the brain, lung and heart of rat [33]. Neokyotorphin was shown to terminate the hibernation state in ground squirrels and to enhance the inward potential dependent Ca^{2+} current in cardiac myocytes of rat [34]; it was not cytotoxic in human erythroid leukaemia (K562) and murine transformed fibroblast (L929) cell lines [33]. In contrast to neokyotorphin, the fragment shortened by one C-terminal amino acid inhibited the Ca^{2+} current [34] and showed pronounced cytotoxic effects in the above-mentioned transformed cell lines [33].

The biological activities of hemorphin-related peptides (LVV-hemorphin-7 (37) [24], VV-hemorphin-7 (41) [24] and valorphin (42) [27]) derived

Table 4 Peptides Originating from Unknown Precursors

No.	Amino acid sequence	Maximum homology[a] (%)	Content (nmol/gram tissue)
70	AYYF[b]	100	0.1–1.0
71	IEG[b]	100	0.1–1.0
72	AVL[b]	100	0.1–1.0
73	EV[b]	100	1.0–3.0
74	VS[b]	100	0.1–1.0
75	LL[b]	100	1.0–3.0
76	VE[b]	100	1.0–3.0
77	SV[b]	100	0.1–1.0
78	KV[b]	100	0.1–1.0
79	PVDNSSP	< 70	0.1–1.0
80	PYVGEIIGKRGIIGY	< 28	0.1–1.0
81	VYYFPG	< 66	< 0.1
82	XXXAEXEQTSAPMV[c]	< 40	< 0.1
83	FEWQLSLMLS	< 80	0.1–1.0
84	LVLFPGK	< 80	0.1–1.0
85	ANKFNKEQ	< 80	< 0.1
86	ALXPPKKG[c]	< 80	< 0.1
87	GQFFE	< 80	0.1–1.0
88	LEPPP	< 80	< 0.1
89	YEAVAL	< 80	0.1–1.0
90	GVFTPP	< 80	< 0.1
91	FEIFQPKVGWNFGG	< 80	0.1–1.0
92	VILXEEKQQRMK[c]	< 80	0.1–1.0
93	FLPXYPARKXKM[c]	< 80	< 0.1
94	FGFQKVP	< 80	< 0.1
95	AAKKAAI	< 80	0.1–1.0
96	IIVPKXQXF[c]	< 80	< 0.1
97	LPPLGXV[c]	< 80	0.1–1.0
98	DDRIRIRNKVSGLNGVPN	< 80	0.1–1.0
99	YQNPATPVPGLQXVPA[c]	< 80	0.1–1.0
100	IQAIVIPNPVSGLQGV	< 80	< 0.1
101	GENPGTQKGFQKYVPA	< 80	< 0.1
102	XVKVKV[c]	< 80	0.1–1.0
103	AQASFPKLQQ	< 80	< 0.1
104	VGLETLGKLK	< 80	< 0.1
105	XATPPGTFY[c]	< 80	< 0.1
106	FGPPHGTVLXXMXNY[c]	< 80	< 0.1
107	VVLFXIXFXXXFXQ[c]	< 80	< 0.1

[a]Percentage of identical amino acid residues at the same positions in the segments of protein sequences from PIR data bank. The length of each protein segment corresponds to that of the analysed peptide.
[b]Sequences present in several proteins.
[c]Unidentified amino acid residues.

from the bovine β-globin 31–40 segment were active in three basic opioid tests: inhibition of opioid ligands binding to brain membranes, induction of naloxone-dependent analgesia in rats *in vivo* and contractile activity at the guinea pig *ileum* [20, 27, 35]. Other effects corresponding to the opioid nature of endogenous haemoglobin fragments belonging to the hemorphin family of peptides (including peptides 37, 41 and 42) were also detected in the literature, such as coronaro-constrictory [36], antitumour [37] and immunoregulatory activities [26], as well as inhibition of angiotensin-converting enzyme [38].

The fragment of immunoglobin (peptide 59 in Table 3) selectively modulates the [^{3}H]-glutamate

binding at 0.01–10 mM concentrations. In behavioural studies, that peptide manifests antiagressogenic activity [39].

The fragment of a multidrug resistance protein (peptide 63 in Table 3) at 3–4 μM concentration inhibited the binding of a ligand that interacts with certain subtypes of serotonin and adrenergic receptors. These data correlate with the effects on the 'head-twitch' phenomenon induced in mice by 2,2-dimethoxy-4-methylphenylisopropylamine, an antagonist of the serotoninergic system. Administered i.p. at 0.05–0.2 mg/kg, the peptide suppressed the 'head-twitch' by 30–40% [39].

Some of the peptides isolated from bovine brain extract (Tables 2 and 3) show a very considerable overlap with known biologically active peptides. Accordingly, peptides (38 and 39) are expected to display opioid properties [40]: peptide (29) – growth hormone release activity *in vivo* and *in vitro* [41]; peptides 30, 34, 35 and 46) – haemopoietic activity (the ability to restore the haemopoietic function of mice subjected to radiation or treated with 5-fluorouracil) [29]; peptide (68) – neurotensin-like activity [42].

Some fragments of functional proteins were shown to act inside the cell. In particular, the fragment of calmodulin-binding protein kinase C substrate neurogranin (peptide 1 in Table 2) exhibited calmodulin-binding activity. It was also a good substrate of pkC [43]. Amino acid sequence of this peptide contains both the Ser residue phosphorylated by pkC and the calmodulin binding site. Neurogranin is a member of a large group of B-50 proteins which includes a number of calmodulin-binding substrates of pkC (F-1, GAP-43, neuromodulin) [44]. All these proteins contain the segment exhibiting a high homology (about 75%) with the neurogranin sequence. Another fragment of GAP-43, PIP, was shown to inhibit phosphorylation of GAP-43 by pkC [45]. It is very possible that the above-mentioned peptides, including the neurogranin fragment (1), participate in intracellular regulation of neurone growth and differentiation.

The fragment (335–349) of glyceraldehyde-phosphate dehydrogenase isolated from bovine brain extract (Table 3, peptide 53) includes, according to X-ray data [46], the segment containing the Tyr residue of the active site of the enzyme as well as the sequence involved in the interaction of enzyme subunits. Accordingly, we assume that this peptide could be involved in regulation of the enzyme activity.

The data collected in this section provide sufficient ground to conclude that several of the newly identified brain peptides should participate in the regulation of extracellular and intracellular biochemical processes.

The Concept of a Tissue-specific Peptide Pool

Having presented the concrete experimental results and their immediate consequences, we attempt in this final section a broader consideration of peptidergic regulatory mechanisms in order to define the place of peptides derived from functional proteins in these mechanisms.

It is well known that each tissue contains a specific set of proteins. The combined action of these proteins ensures the functioning of the given tissue and, as a result, of the whole organism. Components of such protein sets are constantly synthesized by the cells of corresponding tissue, then they exhibit their function, and, at last, they are digested by proteases. We believe that the process of protein elimination is not a random proteolysis which leads in a straightforward manner to amino acid building blocks. Instead, it is carried out by a specific and regulated system of tissue-specific enzymes and protein substrates. This process results in the formation of a large group of peptides that can be defined as the 'peptide buffer' or 'peptide background' of the tissue, or more accurately, as a 'tissue-specific peptide pool'. The composition and content of each component in the pool are characteristic for the given organ or tissue.

As seen above in the example of bovine brain, several components of tissue-specific peptide pool exhibit various biological activities. Some of these activities involve interaction with the receptors of 'classical bioregulators', modulating thereby the availability of these receptors to their 'true' agonists. In contrast to signal molecules of the nervous system (neurotransmitters) and the endocrine system (hormones) which are formed from specific precursors, the components of tissue-specific peptide pools are released from a wide number of proteins possessing other functions.

Neurotransmitters, such as substance P [47] or vasopressin [48, 49], can be released directly into the synaptic cleft. Such peptides are characterized by high binding parameters with their receptors and relatively short time of action (several minutes) [50]. Peptide hormones, such as MSH or VIP, are released from the cells and are distributed by blood (hormonal regulation) or directly influence the neighbouring

cells (paracrine regulation). Peptide hormones are characterized by high affinity of the receptor binding the duration of their direct action in the organism is not more than several hours [51]. If the classical regulatory peptide and the fragment of a functional proteins act on the same receptor the hormone or neurotransmitter-induced signals can be controlled by the level of proteolytic activity. The high content of fragments of functional protein might compensate for the relatively low binding parameters of these peptides. For instance, the binding constant of the fragment of the β-chain of a haemoglobin (31–39) (Table 2, peptide 41) with μ-opioid receptors is two orders of magnitude lower than for Leu-enkephalin (270 nM [20] compared with 1 nM [52]), while the overall content of the + related fragments of β-globin (31–40, 31–38, 31–37, 32–40, 32–38) is as high as *ca.* 3 nmol/g of brain tissue compared with 0.01 nmol/g in the case of Leu-enkephalin [52]. At the same time, endogenous neurotensin-like peptide fragments of functional protein bind to neurotensin receptors with an affinity similar to that of neurotensin [53].

Since the level of the proteolytic activity within the tissue is linked with the metabolic state of the organism, we suggest that the brain tissue-specific peptide pool controls the long-term status of the organism, such as sleep, seasonal rhythms or the immune status [2]. One should also expect that parameters of the peptide pool in the case of pathology should display noteable differences compared with the normal state, as indeed was recently observed for Alzheimer's disease [21] and brain ischaemia [54].

Summarizing the data described above we suggest that fragments of functional proteins form a novel specialized system of peptide regulation. That system is distinct from endocrine or paracrine systems, sharing at the same time a number of similar properties with these systems. The principal characteristics of the three regulatory systems are represented in Table 5.

Components of the tissue-specific peptide pools have several features in common with the so-called 'cytomedines', a term proposed in the early 1980s for the components with low molecular mass fractions of the total tissue extracts. Similarly to the peptide pool, cytomedines were ascribed a homeostatic role for the given tissue (see the review [55] and the references therein). However, most of the properties of cytomedines were studied on complex, poorly characterized mixtures containing all types of regulatory peptides presented in Table 5.

Developing the concept of a tissue-specific peptide pool further, we assume that proteolysis-based peptidergic systems could represent a relatively ancient regulatory system. This proposal follows from the fact that the peptide pool does not necessarily require the entire organism to express its activity. Most of its potential is employed within the isolated tissue. Moreover, some of the above-described examples (fragments of neurogranin and GAP-43) refer to fragments of intracellular proteins regulating a variety of responses of the same cells. In other words, such a mechanism could be applied even to unicellular organisms.

Table 5 Distinctive Features of Peptidergic Regulatory Systems

	Peptidergic regulatory system		
Characteristic	Nervous	Endocrine and paracrine	Tissue-specific peptide pool
Peptides	Neurotransmitters	Hormones	Fragments of functional proteins
Precursor	Specific protein precursor		Functional protein
Type of processing	Discrete site-specific processing		Action of tissue proteinases
Level (pmol/g tissue)	0.001–1.0	0.001–1.0	10–10,000
Type of regulation	Synaptic secretion	Extracellular secretion	Alteration of the level in the tissue
Mechanism of action	Binding to receptors in postsynaptic membrane	Binding to receptors in cellular membranes	Binding to receptors of homologous hormones
Receptor binding constants (K_d, nM)	1–1000	0.1–10	100–10,000
Time range of action	Seconds–minutes	Minutes–hours	Hours–days
Biological role	Transmission of nervous impulse	Regulation physiological processes in the tissue or the whole organism	Maintenance of tissue homeostasis

In higher organisms, the components of a tissue-specific peptide pool might participate in the maintenance of tissue homeostasis, and, in particular, in assisting the hormonal signals in cases when the tissue is unable to receive such signals. We also envision the possibility of inverse regulation, when changes in the efficiency of local proteolytic processes take place as a result of hormonal signals.

The described system of regulation includes protein substrates, proteolytic enzymes and products of their interactions (fragments of these proteins). The large number of components involved in that system inevitably makes it polyfunctional. Considering the high stability of such a system [30], we suggest that in a broad sense its main function is participation in adaptation and compensation mechanisms [2].

CONCLUSIONS

The present paper provides an example of a systematic analysis of animal tissue extract for peptide components. The results obtained allowed a novel concept to be formulated of a peptide-based regulatory system defined as a tissue-specific peptide pool. The described work follows the principles of the so-called 'reverse biology' or 'from structure to function' studies. We believe that modern techniques of separation and structural analysis applied within the framework of such strategy will allow us to obtain in a short time a considerable bulk of new information on peptides participating in regulatory processes. Such information will hopefully provide new leads to a perspective for therapeutical and diagnostic applications.

REFERENCES

1. R. Carraway and S. E. Leeman (1973). The isolation of a new hyptensive peptide, neurotensin, from bovine hypotalami. *J. Biol. Chem. 248*, 6854–6861.
2. V. T. Ivanov, A. A. Karelin, I. I. Mikhaleva, B. V. Vaskovsky, V. I. Sviryaev and I. V. Nazimov (1993). Isolation, structure and properties of novel endogenous peptides. *Soviet J. Bioorgan. Khim. 18*, 677–702.
3. V. T. Ivanov, A. A. Karelin, M. M. Philippova, I. V. Nazimov and V. Z. Pletnev (1997). Hemoglobin as a source of endogenous bioactive peptides. A concept of tissue specific peptide pool. *Biopolym. Peptide Sci.*, **43**, 171–188.
4. J. H. Julliard, T. Shibasaki, N. Ling and R. Guillemin (1980). High-molecular-weight immunoreactive-endorphin in extracts of human placenta is a fragment of immunoglobulin G. *Science 208*, 183–185.
5. R. C. Carraway, S. P. Mitra and K. Muraki (1990). Isolation and structures of xenopsin-related peptides from rat stomach, liver and brain. *Regul. Peptides 29*, 229–239.
6. F. Nyberg, H. Lieberman, L. H. Lindstrom, S. Lyrenas, G. Koch and L. Terenius (1989). Immunoreactive β-casomorphin-8 in cerebrospinal fluid from pregnant and lactating women: Correlation with plasma levels. *J. Clin. Endocrinol. Metabol. 68*, 283–289.
7. M. Comb, P. H. Seeburg, J. Adelman, L. Eiden and E. Herbert (1982). Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA. *Nature 295*, 663–666.
8. H. Kakidani, Y. Fututani, H. Takehashi, M, Noda, Y. Moromoto, T. Hirose, M. Asai, S. Inayama, S, Nakanishi and S. Numa (1982). Cloning and sequence analysis of cDNA for porcine beta-endorphin, dynorphin precursor. *Nature 298*, 245–249.
9. E. Kislauskis, B. Bullock, S. McNiel and P. R. Dobner (1988). The rat gene encoding neurotensin and neuromedin. *J. Biol. Chem. 263*, 4963–4968.
10. J. F. McKelvy, C.-J. Lin and L. Chan in: *Brain Peptides: A New Endocrinology*, A. M. Gotto, Ed., p. 183–196, Elsevier, Amsterdam 1979.
11. K. Kanagawa and H. Matsuo (1984). Purification and complete amino acid sequence of α-human atrial natriuretie polypeptide. *Biochem. Biophys. Res. Commun. 118*, 131–139.
12. C. N. Jones, L. D. Grant and A. J. Prane Jr (1978). Stimulus properties of thyrotropin-releasing hormone. *Psychopharmacology 59*, 217–224.
13. D. L. Margules (1980). β-endorphin: Hormone for the conservation of bodily resources and energy in anticipation. *Soc. Neurosci. 5*, 221.
14. H. P. J. Bennett, R. Y. Chang, L. Nelbach and J. W. Adelson (1990). The amino acid sequence of chemodenin, a hormone-like peptide from porcine duodenum, is identical to cytochrome C-oxidase, peptide VII. *Regul. Peptides 29*, 241–250.
15. J. N. Whitaker, M. Gupta and O. F. Smith (1986). Epitopes of immunoreactive myelin basic protein in human cerebrospinal fluid. *Ann. Neurology 20*, 329–336.
16. V. A. Najjar (1983). Tuftsin, a natural activator of phagocyte cell: An overview. *Ann. NY Acad. Sci. 419*, 1–11.
17. D. E. Cochrane, R. E. Carraway, R. S. Feildberg, W. Boucher and J. M. Gelfand (1993). Stimulated rat mast cells generate histamine-releasing peptide from albumin. *Peptides 14*, 117–123.
18. L. Standker, R. Sillard, K. W. Bensch, A. Ruf, M. Raida, P. Schulz-Knappe, A. G. Schepky, H. Patscheke and W.-G. Forssmann (1995). *In vivo* degradation of human fibrinogen Aα: Detection of cleavage sites and release of antithrombotic peptides. *Biochem. Biophys. Res. Commun. 215*, 896–902.

19. T. Ohmori, T. Nakagami, H. Tanaka and S. Maruyama (1994). Isolation of prolylendopeptidase-inhibiting peptides from bovine brain. *Biochem. Biophys. Res. Commun. 202*, 809–815.
20. E.-L. Glamsta, A. Marclund, U. Hellman, C. Wernstedt, L. Terenius and F. Nyberg (1991). Isolation and characterization of a hemoglobin-derived opioid peptide from the human pituitary gland. *Regul. Peptides 34*, 169–179.
21. J. R. Slemmon, C. M. Hughes, G. A. Cambell and D. G. Flood (1994). Increased levels of hemoglobin-derived and other peptides in Alzheimer's disease cerebellum. *J. Neurosci. 14*, 2225–2235.
22. E.-L. Glamsta, B. Meyerson, J. Silberring, L. Terenius and F. Nyberg (1992). Isolation of a hemoglobin-derived opioid peptide from cerebrospinal fluid of patients with cerebrovascular bleedings. *Biochem. Biophys. Res. Commun. 184*, 1060–1066.
23. K. K. Maekinen, L. Sewon and P. L. Maekinen (1996). Analysis in gingival crevicular fluid of two oligopeptides derived from human hemoglobin β-chain. *J. Periodont. Res. 31*, 43–46.
24. R. C. C. Chang, W.-Y. Huang, T. W. Redding, A. Arimura, D. H. Coy and A. V. Schally (1980). Isolation and structure of several peptides from porcine hypothalmi. *Biochem. Biophys. Acta 685*, 266–273.
25. A. Mikhailova, L. Fonina, E. Kirilina, S. Shanurin, S. Gur'yanov, A. Malakhov, V. Nesmeyanov and R. Petrov (1994). Immunoregulatory properties of hexapeptide isolated from porcine bone marrow cell culture. *Regul. Peptides 53*, 203–209.
26. H. Takagi, H. Shiomi, K. Fukui, K. Hayashi, Y. Kiso and K. Kitagawa (1982). Isolation of a novel analgesic pentapeptide, neo-kyotorphin, from bovine brain. *Life Sci. 31*, 1733–1736.
27. J. Erchegyi, A. J. Kastin, J. E. Zadina and X.-D. Qiu (1992). Isolation of a heptapeptide Val-Val-Tyr-Pro-Trp-Thr-Gln (valorphin) with some opioid activity. *Int. J. Peptide Protein Res. 39*, 477–484.
28. A. A. Karelin, M. M. Philippova, E. V. Karelina and V. T. Ivanov (1994). Isolation of endogenous hemorphin-related hemoglobin fragments from bovine brain. *Biochem. Biophys. Res. Commun. 202*, 410–415.
29. V. T. Ivanov, A. A Karelin, E. V. Karelina, V. V. Ul'yashin, B. V. Vaskovsky, I. I. Mikhaleva, I. V. Nazimov, G. A. Grishina, V. Kh. Khavinson, V. G. Morozov and A. N. Mikhaltsov in: *Peptides, Chemistry and Biology*, J. A. Smith and J. E. River, Eds., p. 939–941, ESCOM, Leiden 1992.
30. J. R. Slemmon and D. G. Flood (1992). Profiling of endogenous brain peptides and small proteins: Methodology, computer-assisted analysis, and applications to aging and lesion models. *Neurobiol. Aging 13*, 649–660.
31. A. A. Karelin, M. M. Philippova and V. T. Ivanov (1995). Proteolytic degradation of hemoglobin in erythrocytes leads to biologically active peptides. *Peptides 16*, 693–697.
32. R. H. Ziganshin, V. I. Sviryaev, B. V. Vaskovsky, I. I. Mikhaleva, V. T. Ivanov, Yu. M. Kokoz, A. E. Alekseev, A. F. Korystova, G. S. Sukhova, T. G. Emelyanova and A. B. Usenko (1994). Bioactive peptides isolated from the brain of hibernating ground squirrel. *Bioorg. Khim. 20*, 899–918 (in Russian).
33. E. Yu. Blishchenko, O. A. Mernenko, O. N. Yatskin, R. H. Ziganshin, M. M. Philippova, A. A. Karelin and V. T. Ivanov (1996). Neokyotorphin and neokyotorphin (1–4): Cytolytic activity and comparative levels in rat tissues. *Biochem. Biophys. Res. Commun. 224*, 721–727.
34. R. H. Ziganshin, I. I. Mikhaleva, V. T. Ivanov, Yu. M. Kokoz, A. E. Alekseev, A. F. Korystova, D. A. Mavlyutova, T. G. Emelyanova and A. K. Akhrimenko in: *Peptides, Chemistry, Structure and Biology*, P. T. P. Kaumaya and R. S. Hodges, Eds., p. 244–245, Mayflower Scientific Ltd, Kingswinford 1995.
35. I. Garreau, Q. Zhao, C. Pejoan, A. Cupo and J.-M. Piot (1995). VV-hemorphin-7 and LVV-hemorphin-7 released during *in vitro* peptic hemoglobin hydrolysis are morphinomimetic peptides. *Neuropeptides 28*, 243–250.
36. N. A. Barkhudaryan, J. Kellermann, A. A. Galoyan and F. Lottspeich (1993). High molecular weight aspartic endopeptidase generates a coronaro-constrictory peptide from the β-chain of hemoglobin. *FEBS Lett. 329*, 215–218.
37. E. Yu. Blishchenko, O. A. Mernenko, I. I. Mirkina, D. K. Satpaev, V. S. Ivanov, L. D. Tchikin, A. G. Ostrovsky, A. A. Karelin and V. T. Ivanov (1997). Tumor cell cytolysis mediated by valorphin – an opioid-like fragment of hemoglobin β-chain. *Peptides 18*, 79–85.
38. Q. Zhao, F. Sannier, I. Garreau, D. Guillochon and J.-M. Piot (1994). Inhibition and inhibition kinetics of angiotensin converting enzyme activity by hemorphins, isolated from a peptic bovine hemoglobin hydrolysate. *Biochem. Biophys. Res. Commun. 204*, 216–223.
39. V. T. Ivanov, A. A Karelin, E. V. Karelina, V. V. Ul'yashin, V. I. Tsetlin, T.N. Alyonycheva, N. V. Nikitin, N. N. Galeva, V. V. Sherstnev, V. I. Spiglazov and N. A. Dimitriadi in: *Peptides, Chemistry and Biology*, J. A. Smith and J. E. River, Eds., p. 157–158, ESCOM, Leiden 1992.
40. J. E. Zadina, D. Poul, K. A. Gergen, L.-J. Ge, L. Hackler and A. J. Kastin (1996). Binding of Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) and related peptides to μ_1 and μ_2 opiate receptors. *Neurosci. Lett. 215*, 65–69.
41. A. V. Schally, Y. Baba and R. M. G. Nair (1971). The amino acid sequence of a peptide with growth hormone-releasing activity isolated from porcine hypotalamus. *J. Biol. Chem. 246*, 6647–6650.
42. R. Carraway and C. F. Ferris (1983). Isolation, biological and chemical characterization, and synthesis of neurotensin-related hexapeptide from chicken intestine. *J. Biol. Chem. 258*, 2475–2479.
43. A. A. Karelin, E. V. Karelina, S. E. Severin Jr, A. N. Gubin and V. T. Ivanov in: *Chemistry of Peptides and*

Proteins, D. Brandenburg, V. Ivanov and W. Voelter, Eds., p. 647–655, Verlag Mainz, Aachen 1993.

44. J. Baudier, J. Ch. Deloulme, A. Van Dorsselaer, D. Black and H. W. D. Matthes (1991). Purification and characterization of a brain specific protein kinase C substrate, neurogranin (p17). *J. Biol. Chem. 266*, 229–237.
45. H. Zwiers, J. Verhoef, P. Schotman and W. H. Gispen (1980). A new phosphorylation-inhibiting peptide (PIP) with behavioural activity from rat brain membranes. *FEBS Lett 112*, 168–172.
46. T. Skarzynski, P. C. Moody and A. J. Wonacott (1987). Structure of hologlyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* at 1.8Å resolution. *J. Mol. Biol. 193*, 171–187.
47. A. M. Khawaja and D. F. Rogers (1996). Tachykinins: Receptor to effector. *J. Biochem. Cell. Biol. 28*, 721–738.
48. D. Albeck, T. Smock, P. McMechen, D. Purves and L. Floyd (1990). Peptidergic transmission in the brain. I. Vasopressin-like signal in hippocampus. *Brain Res. 511*, 7–14.
49. T. Smock, D. Albeck, P. McMechen and D. Purves (1990). Peptidergic transmission in the brain. II. Mediation by vasopressin-like peptide. *Brain Res. 511*, 15–20.
50. G. M. Shepherd in: *Neurobiology*, p. 103–128, Oxford University Press, New York 1983.
51. W. G. Clark, J. M. Lipton and S. I. Said (1978). Hyperthermic responses to vasoactive intestinal polypeptide (VIP) injected into the third central ventricle of cats. *Neuropharmacology 17*, 883–885.
52. G. A. Olson, R. D. Olson and A. J. Kastin (1993). Endogenous opiates: 1992. *Peptides 14*, 1339–1378.
53. R. E. Carraway, S. P. Mitra and D. E. Cochrane (1987). Structure of biologically active neurotensin-related peptide obtained from pepsin treated albumin(s). *J. Biol. Chem. 262*, 5968–5973.
54. J. R. Slemmon, T. M. Wengenack and D. G. Flood (1997). Profiling of endogenous peptides as a tool for studying development and neurological disease. *Biopolymers Peptide Sci.*, **43**, 157–170.
55. R. I. Kuznik, V. G. Morozov and V. Kh. Khavinson (1995). Cytomedines and their role in regulation of physiological functions. *Uspekhi sovremennoi biologii 115*, 353–367 (in Russian).

