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β-Actin-derived peptides isolated from acidic extract of rat spleen suppress tumor cell growth

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Abstract: Twenty-two fragments of β-actin and β-actin-related protein were isolated from the acidic extracts of rat spleen tissue. β-Actin fragments (75–90), (78–89), and (78–88), 0.01–1 μM, decreased live cell number of L929 murine tumor fibroblasts by 80–90%, with maximal cytotoxic effect of 30–40%. The fragments of (78–90) segment and the fragment of β-actin-related protein (69–77) were less active (inhibitory effect up to 55%, cytotoxic–up to 25%). Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: proteolysis; protein fragment; tumor cells; cytotoxicity; antiproliferative effect

INTRODUCTION

Cellular actin constitutes 5–20% of cell protein by weight for nonmuscle cells [1]. The β- and γ-actins coexist in most cell types as components of the cytoskeleton and as mediators of internal cell motility [1]. In addition, actin plays an important role in signal transduction, e.g. progression through G1 phase and apoptosis. Mediators of growth factor-induced signal transduction such as phospholipase C, phosphotidylinositol kinase and others are associated with actin filaments [2]. EGF receptors bind actin with no other proteins involved. Disruption of actin filaments interferes with activation and translocation of mitogen-activated protein kinase Erk1/2. Monomeric G-actin binds to and inhibits the DNA-degrading activity of DNase I [3]; actin hyperpolymerization, cleavage with proapoptotic proteases or gelsolin-induced destabilization of the actin/DNAase I complex result in the enzyme activation [3–5]. Recent findings point that the actin cytoskeleton has a role in regulating apoptosis via interactions with the mitochondria and has a significant impact on the management of oxidative stress [6]. Cleavage of actin by caspase-3 and caspase-1 contributes to morphological changes during execution phase of apoptosis [7,8]. Caspase-3 generates *N*-terminal 32 kDa and *C*-terminal 14 kDa actin fragments detected *in vivo* in degenerating tissues [7–9]. Caspase-1 cleaves actin at three positions (Asp^{11}–Asn^{12}, Glu^{107}–Ala^{108}, Asp^{244}–Gly^{245}) [4].

Extracellular actin released as consequence of trauma/burn and not scavenged by gelsolin induces pathological conditions such as endothelial injury, respiratory distress syndrome, hepatic necrosis and septic shock [10]. Exogenously added monomeric G-actin is toxic for cultured endothelial cells [11].

On the basis of the numerous interactions of actin with key intracellular regulators, it could be suggested that the fragments of those protein should interfere with signal transduction.

Earlier we have reported on the isolation of biologically active protein fragments from acidic extracts of rat tissues [12,13]. The major part of those peptides are derived from hemoglobin. Notwithstanding the abundance of actin, its degradation products were not found in mammalian brain [13,14] or rat lung and heart extracts [15]. On the other hand, two β-actin fragments have been detected in supernatant of human erythrocytes in a surviving culture [16].

In the present work, we report isolation of 22 fragments of β-actin and β-actin- related protein from rat spleen extracts. The isolated peptides, 6–23 amino-acid residues in length, are cleaved from segment 67–90 of the precursor proteins. The isolated peptides were tested for the ability to suppress tumor cell growth and viability.

MATERIALS AND METHODS

Animals

Rats (Wistar, females, 250–300 g) were purchased from Animals Department of the title Institute. The animals obtained standard alimentation and water *ad libitum*.

Abbreviations: As recommended in *J. Pept. Sci.* 2006; 12: 1–12, with following additions: EGF, epidermal growth factor; TNF, tumor necrosis factor; ACE, angiotensin converting enzyme; AT4, angiotensin IV.

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Peptide Extraction

Rats were killed by decapitation. Spleens were isolated within 1 min and immediately freezed and stored in liquid nitrogen. Portions of the spleen tissue (4–6 g) were homogenized in a Potter homogenizer in 10% acetic acid (10 ml per 1 g of tissue, 10 min, 4 °C). The homogenates were centrifuged (2500 g, 10 min, 4 °C, TJ-6 model, Beckman, US). Supernatants were lyophilized.

Size Exclusion Chromatography

The fractionation was performed using Sephadex 25 sf column (2.5 × 85 cm) (Pharmacia, Sweden) equilibrated with 0.1 M acetic acid. Four fractions were collected manually according to the calibrated elution volume: fraction I (2.5–4.5 kDa), fraction II (1.5–2.5 kDa), fraction III (0.2–1.5 kDa), and fraction IV (<0.2 kDa and matrix-adsorbed components). The fractions were lyophilized.

RP-HPLC Separation

Primary separation of the fractions obtained by size-exclusion chromatography was carried out on a Nucleosil 7C8 (10 × 250 mm) column (Macherey–Nagel, Germany) in a linear water-acetonitrile gradient (0–50% of acetonitrile for 100 min, flow rate of 2.5 ml/min) containing 0.1% TFA. Fractions were collected according to the elution time (1–2 min for each fraction), lyophilized and rechromatographed. Rechromatography was performed in two different chromatographic systems: (i) Nucleosil 120-5C8 (4.0 × 250 mm) cartridge column, linear water–acetonitrile gradient (20–52% of acetonitrile for 30 min, flow rate of 1 ml/min), 50 mM sodium acetate buffer (NaOAc), pH 4.5; (2) Nucleosil 120-5C18 (4.0 × 250 mm) cartridge column, linear water–acetonitrile gradient (20–60% of acetonitrile for 30 min, flow rate of 1 ml/min), 0.1% TFA. The elution profiles were recorded at 226 nm with a digitizer (Ampersand, Russia), 1000 mV signal corresponds to 1 optical unit per full scale. Elution profile analysis was performed with MultiChrom–Spectr program, version 2.67 (Ampersand, Russia).

Peptide Sequence Analysis

Amino acid sequences were determined with gas-phase sequencer (Model 477A, Applied Biosystems, US). Identification of the protein precursor was performed using PIR data bank (version 38).

Cell Culture

L929 murine tumor fibroblasts were from the title institute culture collection (originally ATCC). The cells were cultured as described in [17], in RPMI–1640 culture medium (Gibco BRL, GB) supplied with 10% of FBS (Gibco), 2 mM L-glutamine (Sigma, US), 10% of MEM vitamine solution (Flow Laboratories, GB), 100 nM penicillin and 100 μg/ml of streptomycine (Gibco).

Cell Count

The concentration of live and dead cells was determined by visual cell count in hemocytometer in the presence of Trypan blue dye as described in [17]. Percentage of dead cells in each well was calculated. Spontaneous cell death did not exceed 5%.

Statistical Processing of the Results

The statistical significance of the data obtained in 5–6 independent experiments was evaluated as in [17]. For comparison of the effects, unpaired Student's t-test was applied.

RESULTS

Isolation of Peptides

The procedures applied for the isolation of peptides did not exclude the activation of tissue proteases active at acidic pH. In several independent experiments, 0.4–0.8 g of lyophilisate were obtained.

300–400 mg of lyophilized extracts were subjected to size-exclusion chromatography. Four fractions were collected: fraction I (2.5–4.5 kDa, 7.8 ± 0.1 mg/g), fraction II (1.5–2.5 kDa, 6.4 ± 0.3 mg/g), fraction III (0.2–1.5 kDa, 12.5 ± 0.1 mg/g) and fraction IV (<0.2 kDa and matrix-adsorbed components, 65.3 ± 0.1 mg/g). A typical elution profile is given in Figure 1.

Individual components were isolated by 3 rounds of RP-HPLC separation of the fractions obtained after size-exclusion chromatography. The isolated compounds were sequenced by automatic Edman degradation. As a total, 104 peptides cleaved from functional proteins were detected, including 35 hemoglobin fragments (not shown). Fragments of β-actin and β-actin-related protein were detected in fractions III and IV. In fraction IV some of them were coeluted with hemorphins

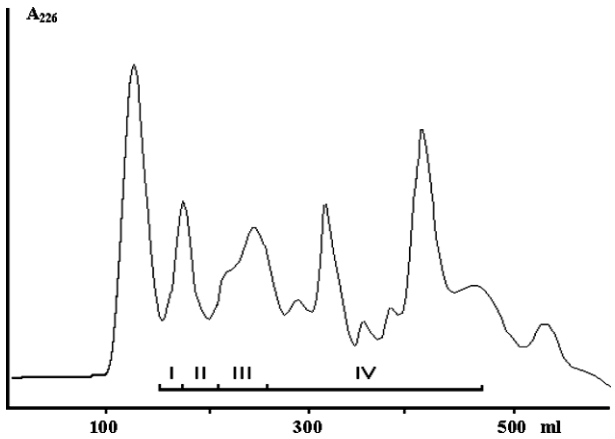

Figure 1 Size-exclusion fractionation of acidic rat speen extract (Sephadex 25 sf column, 2.5 × 85 cm, equilibrated with 0.1 M acetic acid). Collected fractions are marked. The collection was performed manually according to the calibrated elution volume: fraction I (2.5–4.5 kDa), fraction II (1.5–2.5 kDa), fraction III (0.2–1.5 kDa), and fraction IV (<0.2 kDa and matrix-adsorbed components).

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whose isolation from rat spleen extract was reported earlier [12]. Typical elution profiles are shown (Figure 2, chromatography, Figure 3, rechromatography).

In summary, 17 peptides corresponding to rat β-actin fragments and five peptides corresponding to a β-actin-related protein (His^{72} in β-actin is substituted with Arg in β-actin-related protein) were established (Table 1). The level of all peptides was less than 1 nmole/g of a tissue, except for the two longest fragments, β-actin/β-actin-related protein (67–89), whose content was about 2.6 and 1.7 nmole/mg of a tissue, respectively.

Suppression of L929 Live Cell Number by the Peptides

Nine peptides representing various variants of β-actin degradation were tested for the ability to suppress tumor cell culture growth, assayed as reduction of live cell number in the samples treated with test substance, as compared with nontreated controls (Table 2). Epirubicin, a known cytostatic agent inducing DNA damage and cell cycle arrest in L929 cells [18], was used as a reference. The longest and the shortest β-actin fragments [(67–89) and (80–87)]

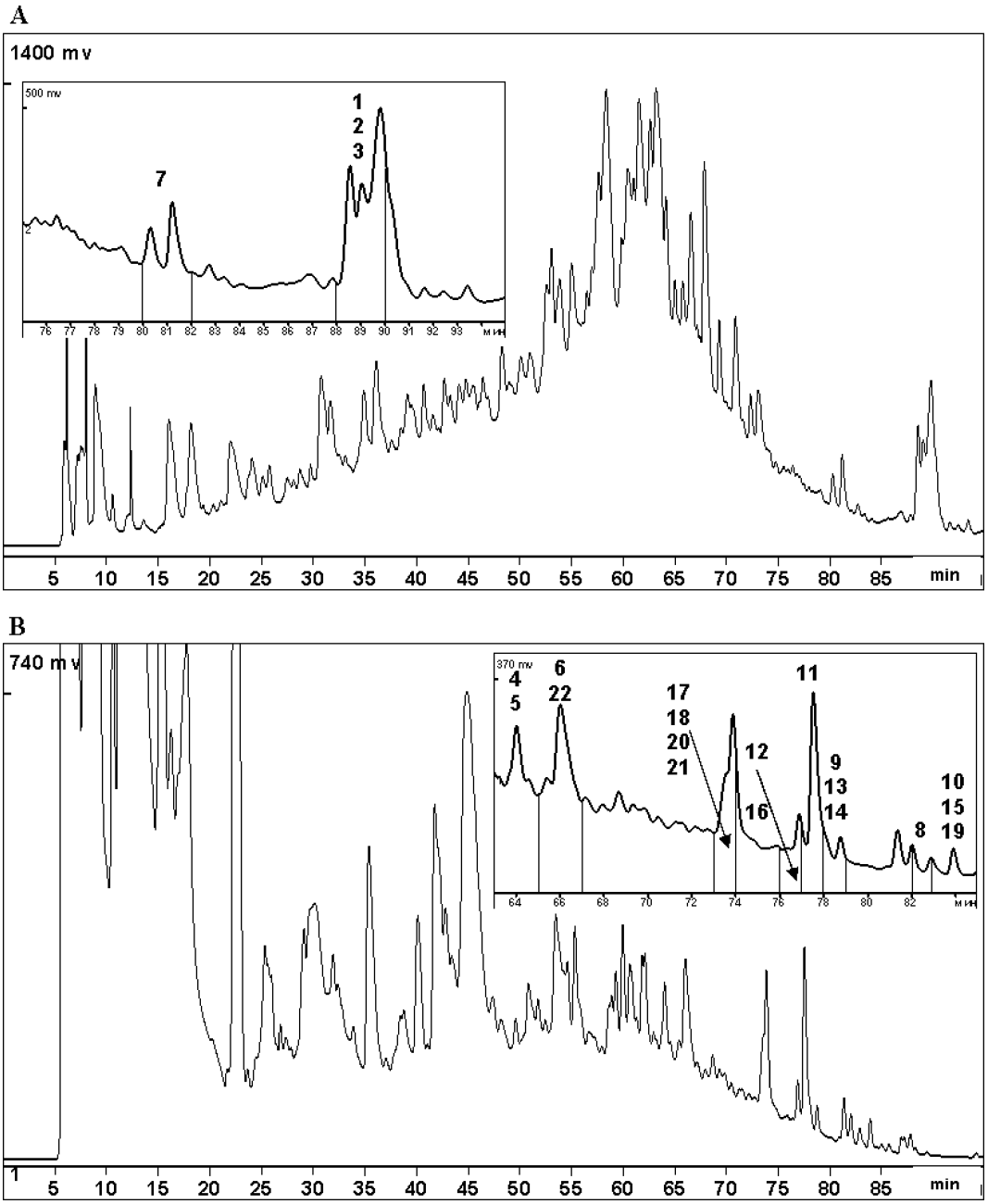

Figure 2 RP-HPLC of fraction III (0.2–1.5 kDa) and fraction IV (<0.2 kDa and matrix-adsorbed components). Nucleosil 7C8 (10 × 250 mm) column (Macherey–Nagel, Germany), linear water–acetonitrile gradient (0–50% of acetonitrile for 100 min, flow rate of 2.5 ml/min), 0.1% TFA. In boxes: elution areas of β-actin/β-actin-related protein fragments. Numbers correspond to numbers of isolated peptides given in Table 1.

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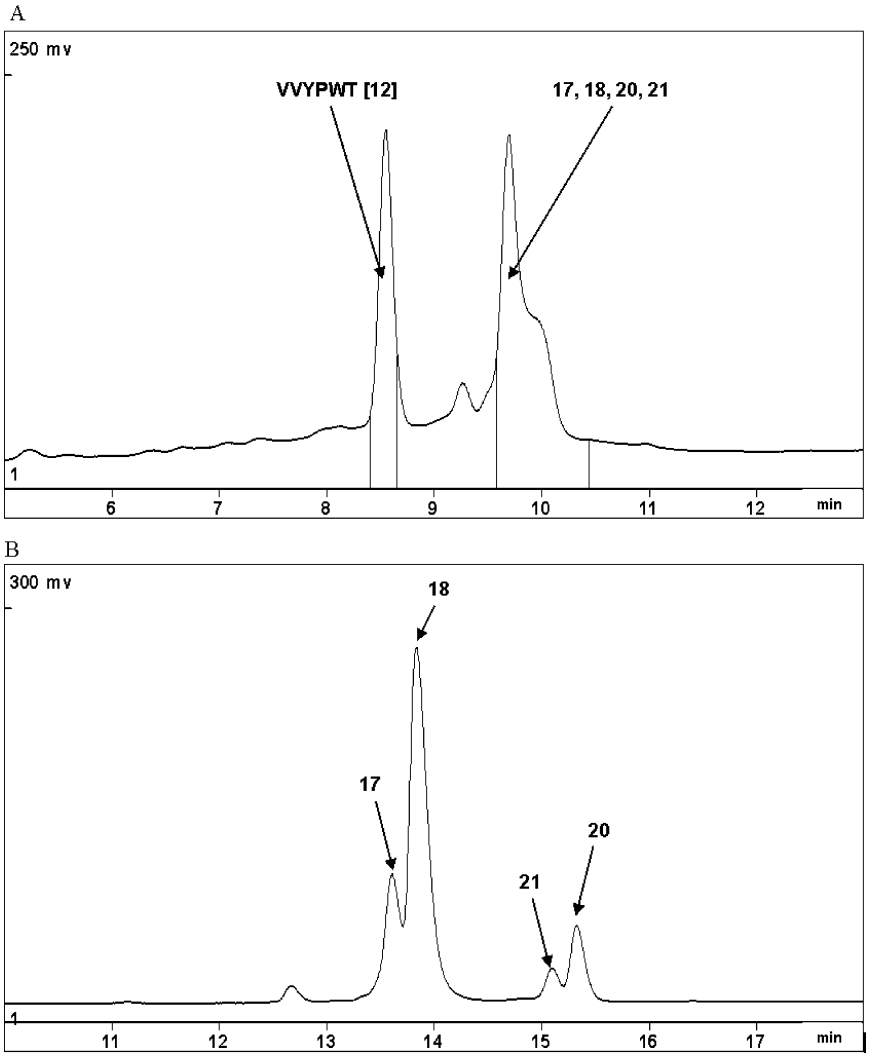

Figure 3 Example of unresolved peak rechromatography: (A) Nucleosil 120-5C8 (4.0 × 250 mm) cartridge column, linear water–acetonitrile gradient (20–52% of acetonitrile for 30 min, flow rate of 1 ml/min), 50 mM sodium acetate buffer (NaOAc), pH 4.5; (B) Nucleosil 120-5C18 (4.0 × 250 mm) cartridge column, linear water–acetonitrile gradient (20–60% of acetonitrile for 30 min, flow rate of 1 ml/min), 0.1% TFA. Numbers correspond to numbers of isolated peptides given in Table 1.

exhibited moderate effect, significantly lower than in epirubicin ($p < 0.05$). In contrast, β-actin fragments (75–90), (78–89), and (78–88) strongly suppressed live cell number (1.5–2-fold stronger than epirubicin, $p < 0.01$). The rest of the tested peptides showed effects close to epirubicin (33–53%). Thus, truncation of the sequence of β-actin (78–88), NWDDMEKIWHH, from *N*- or *C*-terminal results in a significant reduction of the activity in the fragments (78–87), (79–89), (79–87), and (80–87), as compared to three most active peptides which include that segment ($p < 0.01$ for all pairs of the peptides in equal concentrations, except for β-actin (78–89)/(79–89) pair at 0.1 μM, where $p = 0.05$).

Cytotoxic Effect of β-Actin Fragments

Seven β-actin fragments were tested for the ability to induce cell death in L929 cells. Tumor necrosis factor alpha (TNFα), a cytokine shown to induce cytolysis in L929 cells [19], was used as a reference cytotoxic agent. As seen from Figure 4, TNF induced two maxima of cytotoxicity in the concentration range from 1 μM to 0.1 nM: 28% at 0.1 μM and 27% at 0.1 nM. Of the β-actin fragments tested, peptides NWDDMEKIWHHT (78–89) and NWDDMEKIWHH (78–88) had two cytotoxicity maxima. β-Actin (78–88) had a shape similar to TNFα of the concentration/effect dependence with less pronounced maximum at 0.1 nM, and greater effect at 0.1 μM (41%, $p = 0.057$). β-Actin fragment (75–90) exhibited maximal cytotoxic effect close to those of TNFα with one maximum at 1 μM. As in the previous test, the truncation of NWDDMEKIWHH sequence resulted in more or less significant decrease of the maximal cytotoxicity of the resultant peptides {$p < 0.05$ for the pairs β-actin (78–88) *vs* (78–87) and (78–88) *vs* (79–87); $p = 0.06$ for β-actin (78–89) *vs* (79–89)}.

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Table 1 Fragments of β-actin and β-actin-related protein isolated from acidic extract of rat spleen

#	Sequence	Precursor protein		Content, nmole/mg of a tissue
		Name, homology	Position	
1	LKYPIEHGIVTNWDDMEKIWHHT	β-actin, rat, 100%	67–89[a]	2.4–2.8
2	LKYPIERGIVTNWDDMEKIWHHT	β-actin, rat, 97%	67–89	1.6–1.8
3	YPIERGIVTNWDDMEKIWHHT	β-actin, rat, 96%	69–89	0.8–1.0
4	YPIERGIVT	β-actin, rat, 90%	69–77	0.6–0.8
5	YPIEHGIVT	β-actin, rat, 100%	69–77	0.2–0.3
6	YPIERGIV	β-actin, rat, 85%	69–76	0.02–0.03
7	RGIVTNWDDMEKIWHHT	β-actin, rat, 95%	73–89	0.3–0.4
8	IVTNWDDMEKIWHHTF	β-actin, rat, 100%	75–90	0.2–0.3
9	VTNWDDMEKIWHHT	β-actin, rat, 100%	76–89	0.2–0.3
10	NWDDMEKIWHHTF	β-actin, rat, 100%	78–90	0.02–0.04
11	NWDDMEKIWHHT	β-actin, rat, 100%	78–89	0.8–1.0
12	NWDDMEKIWHH	β-actin, rat, 100%	78–88	0.2–0.3
13	NWDDMEKIWH	β-actin, rat, 100%	78–87	0.05–0.1
14	NWDDMEKIW	β-actin, rat, 100%	78–86	0.1–0.2
15	WDDMEKIWHHTF	β-actin, rat, 100%	79–90	0.1–0.2
16	WDDMEKIWHHT	β-actin, rat, 100%	79–89	0.4–0.6
17	WDDMEKIWHH	β-actin, rat, 100%	79–88	0.4–0.5
18	WDDMEKIWH	β-actin, rat, 100%	79–87	0.1–0.2
19	DDMEKIWHHT	β-actin, rat, 100%	80–89	<0.01
20	DDMEKWHH	β-actin, rat, 100%	80–88	0.1–0.2
21	DDMEKIWH	β-actin, rat, 100%	80–87	<0.01
22	EKIWHHTF	β-actin, rat, 100%	83–90	0.1–0.2

[a] The sequences include *N*-terminal Met, as in PIR database (http://pir.georgetown.edu).

Table 2 Reduction of tumor cell number induced by fragments of β-actin and β-actin-related protein. L929 cells were incubated with test substances as described in Figure 4 legend. Live cell concentration (cells/ml) was determined as indicated in Materials and Methods. Reduction of live cell number was calculated as follows: Reduction (%) = $[(C_{cont} - C_{exp})/C_{contr}] \times 100\%$, where C_{exp} is live cells concentration in an experimental sample; C_{contr} is mean live cell concentration in negative control samples

Test substance	Live cell number reduction (%) as compared to negative control				
	1 μM	0.1 μM	0.01 μM	1 nM	0.1 nM
β-actin (67–89)	$24 \pm 3^{*}$	24 ± 6	10 ± 5	3 ± 4	NT
β-actin (75–90)	$86 \pm 6^{**}$	$80 \pm 4^{**}$	$90 \pm 5^{**}$	$48 \pm 12^{*}$	$58 \pm 16^{*}$
β-actin (78–89)	$87 \pm 5^{**}$	$80 \pm 5^{**}$	$92 \pm 4^{**}$	$69 \pm 10^{**}$	$45 \pm 12^{*}$
β-actin (78–88)	$88 \pm 4^{**}$	$85 \pm 4^{**}$	$74 \pm 8^{**}$	$66 \pm 8^{**}$	$48 \pm 10^{*}$
β-actin (78–87)	$37 \pm 9^{**}$	$54 \pm 8^{**}$	$39 \pm 8^{**}$	$31 \pm 10^{*}$	NT
β-actin (79–89)	$44 \pm 7^{**}$	$57 \pm 11^{**}$	$42 \pm 10^{*}$	16 ± 12	18 ± 4
β-actin (79–87)	$33 \pm 5^{**}$	$24 \pm 8^{*}$	0 ± 8	0 ± 6	NT
β-actin (80–87)	$24 \pm 3^{*}$	$28 \pm 6^{*}$	6 ± 6	4 ± 1	6 ± 3
β-actin (68–76)	$53 \pm 5^{**}$	$45 \pm 3^{**}$	$42 \pm 3^{**}$	$41 \pm 6^{**}$	NT
Epirubicin	$53 \pm 8^{**}$	NT	NT	NT	NT

NT = not tested.
* $p \leq 0.05$ *versus* negative control.
** $p \leq 0.01$ *versus* negative control.

DISCUSSION

The fragments of β-actin and β-actin-related protein isolated in the present work are with high probability post-mortem proteolysis products. As no protease inhibitors were utilized during peptide extraction, acidic proteases, e. g. cathepsins, could contribute to protein degradation. Our results where we analyze tissue peptides isolated in the presence of protease inhibitors [20] as well as the literature data [21] indicate the

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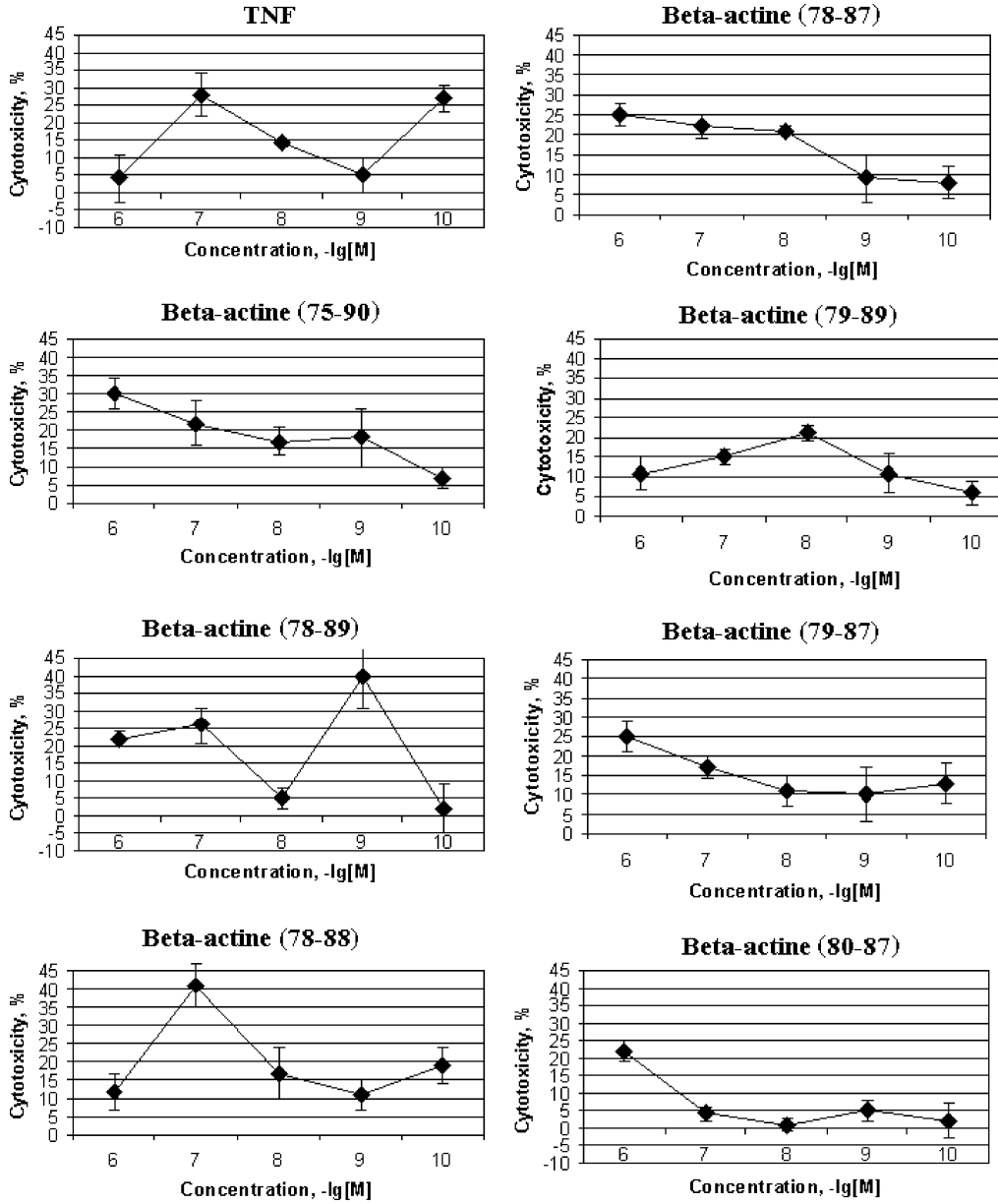

Figure 4 Cytotoxic action of β-actin fragments. L929 cells were placed in 96-well plates (10 000 cells/well) and incubated with test peptides or TNFα (Sigma) dissolved in serum-free culture medium for 18–24 h. Nontreated cells were used as a negative control. Dead cells percentage was determined as indicated in Materials and Methods. Cytotoxicity was calculated as follows: cytotoxicity, (%) = [dead cells % in a sample] − [mean dead cells % in negative control].

immediate activation of tissue proteases upon the tissue disintegration. On the other hand, two fragments of β-actin, namely, KYPIEHGIVT and WDDMEK were detected in the supernatant of human erythrocytes [16], pointing to the possibility of endogenous generation of extracellular actin fragments.

Fragments of β-actin and its related protein found in spleen extract are generated by patterns similar to hemoglobin degradation pathway in erythrocytes [22] or generation of hemorphins in acidic mammalian tissue extracts [12]. On the basis of the structure and the level of the peptides isolated from spleen, the majority of the peptides (17 of 22) arise from primary splitting of precursor actin chain at Thr *C*-termini, namely Thr^{66}, Thr^{77} and Thr^{89}, followed by *N*- and *C*-terminal degradation of the resultant peptides.

The fragments of β-actin and its analog exhibited pronounced suppressive effect on tumor cell growth and viability. It is widely admitted that free extracellular actin found in plasma post-trauma/burn induces pathological conditions in organism [10]. The exogenously added G-actin suppresses viability in three types of cultured endothelial cells after 4 h of incubation with effect linearly depending on concentration [11]. β-Actin fragments tested for toxicity in the present work exhibited moderate toxicity with nonlinear effect dependence

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on concentration, indicating that their mechanism of action is different from G-actin toxicity in endothelial cells.

As mentioned above, actin fragments are not unique bioactive peptides isolated from tissue extracts. There is accumulating evidence that proteolytic degradation of functional proteins gives rise to a variety of peptides with functions and/or effects distinct from their parent proteins [15,23–25]. Digestion of proteins with various proteases (e.g. pepsin, trypsin, thermolysin) yields short (2–20 amino acid residues) biologically active peptides exhibiting diverse effects, ACE I inhibition being the most common case. Among ACE I peptide inhibitors are human serum albumin and α_2-macroglobulin fragments [26,27], hemoglobin α- and β-chain fragments [28–30]. Four cytoplasmic actin fragments inhibiting ACE I were obtained from bonito muscle digest [31], of the peptide IWHHT and its synthesized fragment IW inhibited ACE I with IC50 2–5 μM.

The other effects exhibited by short fragments of proteins are radical scavenging (fragments of myofibrillar proteins, e.g. myosin, tropomyosin, microtubule-associated protein [32], collagen/gelatin fragments [33,34]), apoptotic action (lactoferrin fragments [35]), opiate agonism/antagonism (hemorphins, [36]), inhibition of cathepsin B (fragments of plasma proteins, e.g. immunoglobulin G, transferrin [37]), immunomodulation (fibronectin fragments [38]).

Hemoglobin, the protein most abundant in vertebrates, has been shown to encrypt the record number of biologically active peptides. To date, for more than 50 hemoglobin fragments at least one type of activity is reported, among which are antibacterial effect, analgesia/opiate agonism/antagonism [36], vasodilation/ACE I inhibition [39], bradykinin potentiation, enzyme inhibition [40], AT4 (angiotensin IV) receptor interaction [41] and some others. About 75% of hemoglobin fragments tested in L929 cells affected their proliferation/viability [15]. Of them, peptides belonging to hemorphin structure family exhibited effects close to those of β-actin fragments, i. e. pronounced cell number suppression associated with cytotoxicity [42]. As with hemorphins, the effectiveness of proliferation/viability suppression by β-actin fragments depended on the degree of peptide chain degradation. In the case of actin, cleavage of nonactive fragment (67–89) resulted in formation of two families of highly active peptides overlapping segments (69–77) and (75–90). Trimming of the fragment (78–88) led to significant decrease of the suppressive action in the resultant peptides. Analogously to hemorphins, the observed values of live cell number decrease by β-actin fragments correlated with their cytotoxic activity. The detailed study of VV-hemorphin-5 (valorphin) action in cell cultures [43] has demonstrated that this peptide induces both cytotoxicity and reversible cell cycle arrest. We believe that suppressive action of β-actin fragments is also due both to proliferation arrest and induction of cell death. The mechanism of suppressive action of β-actin fragments could be due to their interference with actin-involving signal transduction pathways that results in proliferation arrest and induction of apoptosis.

One could speculate that the presence of growth suppressive peptides within the sequences of abundant functional proteins is a part of the defense against pathogens or uncontrolled cell proliferation. The massive utilization of such defense mechanisms could be restricted to organisms that lack specific immune system, though the active sequences stay encrypted in the proteins with conservative structures present in higher organisms. Cytoplasmic actin is a good example of such proteins, in particular, actin fragment 67–89 is common in animals, including protozoa and invertebrates. In some cases, fragments of proteins could still serve as a part of defense system in higher organisms, e.g. there are data on the antimicrobial action of hemoglobin fragments (hemocidins [44]) and fragments of histones [45] in mammals.

Summarizing, we believe that screening of degradation products derived from abundant proteins is a promising approach to discovery of novel active peptides.

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