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BIOLOGICAL ACTIVITY OF THE PROTEIN COMPLEX FROM SOY MEAL

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The amino-acid composition of the active fraction of a protein complex was determined. An analysis of the N-terminal amino acids found threonine, glycine, serine, proline, and valine at N-terminuses. The protein complex at a dose of 100 μg/mL suppressed incorporation of ^{14}C-thymidine marker by 64.0 ± 1.9% in a radiometric cytotoxicology test for KML murine melanoma cell proliferation. The protein complex contained protease inhibitors, inhibited the growth of tumor strain AKATON by greater than 60%, and did not inhibit bone marrow cell proliferation.

Keywords: protein complex, KML murine melanoma cell line, protease inhibitors, proliferation, apoptosis.

Protein inhibitors of proteases, which delay the invasion and metastasis of malignant tumors, are currently under intense scrutiny [1]. Protease inhibitors are found in all bean cultures including peas [2]. The very low-molecular-weight protease inhibitor of Bowman–Birk with molecular weight (MW) 8 kDa is currently the most studied among the various inhibitors [3]. Even partially purified protease inhibitors are effective because protein impurities protect the inhibitors by competing for proteases present in the GI tract [4].

Scientists from the Chemistry Department of Lomonosov Moscow State University are studying the therapeutic effects of soy proteins. The Insol drug prepared by them exhibits anti-inflammatory and anticancer properties and consists of soybean extract that contains a Bowman–Birk-type protease inhibitor, soy proteins, and an undetermined type of carbohydrate that is currently under investigation in the laboratory [5].

Our goal was to isolate proteins from soy meal in order to investigate their biological activity.

Proteins were isolated using Tris buffer (0.05M, pH 7.4). A model test system for selecting antitumor compounds *in vitro* against KML murine melanoma cell line (B-16 murine melanoma strain) was used for testing the antitumor activity of the proteins.

A determination of the activity of the fractions according to ^{14}C-thymidine incorporation showed that the substance was active. The substance was considered active if at a dose of 100 μg/mL it suppressed KML cell growth by 50% and greater [6]. The suppression of KML cell proliferation at a dose of 100 μg/mL was 52.3 ± 1.4% (Table 1).

Further purification by elution from Sephadex G-75 produced three fractions. Figure 1 shows the gel-filtration chromatogram.

A determination of the activity of the fractions according to ^{14}C-thymidine incorporation showed that the first fraction was the most active. The suppression of KML cell proliferation was 58.5 ± 1.4% at a dose of 100 μg/mL (Table 1).

The first fraction included principal polypeptides with MW 30, 32, and 35 kDa (Fig. 2) that were missing in the second and third fractions.

Further purification of active fraction I that was obtained from the Sephadex G-75 column used an AcA34 ultragel column and NaCl (0.5M) at pH 7.4

Figure 3 shows the gel-filtration chromatogram. A determination of the activity of the fractions according to ^{14}C-thymidine incorporation showed that the active fraction was the second peak that eluted. The suppression of marker incorporation was 64.0 ± 1.9% at a dose of 100 μg/mL (Table 1).

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TABLE 1. Activity of Fractions According to ^{14}C-Thymidine Incorporation

Protein fraction	Protein content, %	Suppression of ^{14}C-thymidine incorporation into DNA, %
Initial fraction	18.2 ± 0.6	52.3 ± 1.4
	Fractions from Sephadex G-75 column	
1	22.5 ± 0.17	58.5 ± 1.4
2	16.4 ± 0.06	11.1 ± 1.5
3	8.2 ± 0.12	6.9 ± 1.6
	Fractions from AcA34 ultragel column	
1	12.1 ± 0.06	1.2 ± 0.6
2	18.0 ± 0.12	64.0 ± 1.9
3	16.4 ± 0.17	8.8 ± 0.6
4	9.2 ± 0.12	3.0 ± 0.7

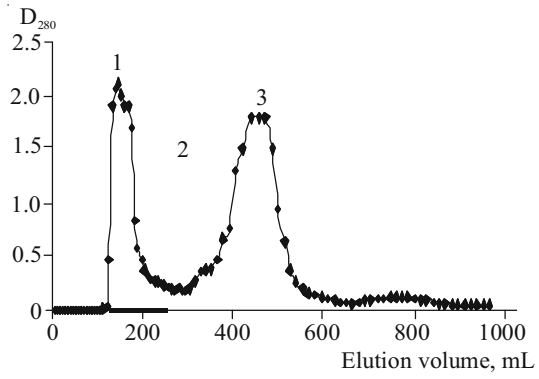

Fig. 1.

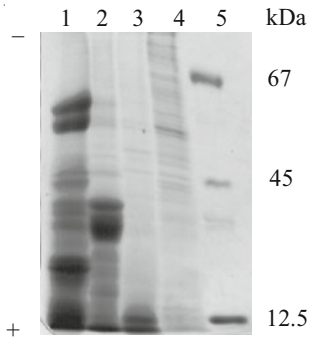

Fig. 2

Fig. 1. Gel-filtration of protein from soy meal on Sephadex G-75 (80 × 2.6), 0.05M Tris-HCl buffer, pH 7.4.

Fig. 2. Electrophoresis in PAAG gradient (10–15%) in the presence of SDS of protein from soy meal on Sephadex G-75. Initial protein fraction (1), fraction I (2), fraction II (3), fraction III (4), mixture of marker proteins (BSA, 67 kDa; ovalbumin, 45 kDa; cytochrome C, 12.5 kDa).

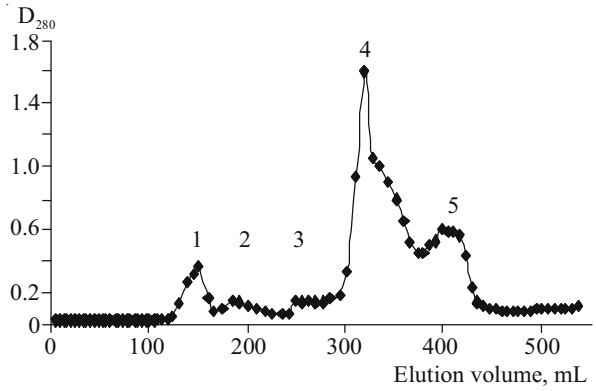

Fig. 3.

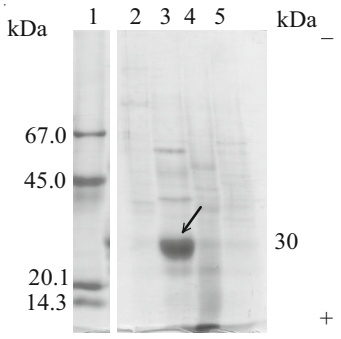

Fig. 4

Fig. 3. Gel-filtration of protein complex on AcA34 ultragel (80 × 2.5), NaCl (0.5M), pH 7.4.

Fig. 4. Electrophoresis in PAAG gradient (10–15%) in the presence of SDS of protein from soy meal after separation on AcA34 ultragel. Mixture of marker proteins (BSA, 67 kDa; ovalbumin, 45 kDa; soy trypsin inhibitor, 20.1 kDa; lysozyme, 14.3 kDa) (1); fraction I (2), fraction II (3), fraction III (4), fraction IV (5).

According to gel-electrophoresis, the active fraction consisted of a complex of proteins with a principal component of MW 30 kDa (Fig. 4).

TABLE 2. Inhibition of Trypsin Protease Activity by Protein sp-2

Protein fraction sp-2	A_{280}	Inhibition, %
Total fraction	0.145 ± 0.002	34.7 ± 0.1
Fraction I after gel-filtration on Sephadex G-75 column	0.156 ± 0.003	29.4 ± 0.2
Fraction II after gel-filtration on AcA34 ultragel column	0.167 ± 0.002	24.1 ± 0.1
Control*	0.220 ± 0.002	0

*3.0 mg trypsin in phosphate buffer (0.05M, pH 7.6); substrate casein (300.0 μg), optical density measured at 280 nm.

The percent content of the principal component with MW 30 kDa increased by six times as a result of the purification (from 5.36 to 29.16%). The activity increase of the protein complex was probably due to the increase in the percent content of this polypeptide.

Next isofocusing determined the isoelectric point of the most active protein fraction. Three components were observed with isoelectric points in the range pI 3.8, 3.9, and 8.2.

The amino-acid composition of the active protein fraction is given below:

Amino acid	*Content, %*	*Amino acid*	*Content, %*
Ala	5.30	Leu	9.04
Arg	10.08	Lys	4.90
Asp	11.09	Met	1.40
Val	5.06	Pro	5.19
His	3.57	Ser	4.85
Glu	16.03	Tyr	3.09
Gly	4.05	Thr	4.90
Ile	3.01	Phe	4.05

A determination of the *N*-terminal amino acids of the active fraction found five *N*-terminuses, threonine, glycine, serine, proline, and valine.

The active protein complex of five polypeptides that was eluted as the second fraction from AcA34 ultragel was called protein sp-2 (soy protein).

Serine proteases containing serine at the active site (trypsin, chymotrypsin, callicrein, plasmin, thrombin) and metalloproteinases containing Zn^{2+} at the active site play an important role in tumor growth [7, 8]. Therefore, we determined the antitrypsin activity of sp-2. Table 2 presents the results. It can be seen that sp-2 exhibits an ability to inhibit trypsin that decreases by 1.4 times after purification. This suggested that it contained protease inhibitors in its complex.

According to the literature [1], protein inhibitors of proteases have a high affinity for proteases and are effective at low concentrations. Various synthetic protease inhibitors also exhibit anticancer properties [9]. However, the chemical inhibitors have a low affinity for proteases. Therefore, a high concentration of the chemical inhibitors is required in order to use them *in vivo*. This is fraught with complications and high consumption for treatment. Therefore, it is preferable to use protein inhibitors of proteases. Protease inhibitor drugs are promising for use after radical surgical excision of malignant tumors in order to prevent relapse and metastasis [1].

The toxicity of sp-2 at three doses (150, 500, and 1000 mg/kg, *per os* administration) was tested for 60 d before studying its antitumor activity *in vivo*. The parameters of internal organs were studied in experimental animals. It was observed visually that brain, heart, liver, spleen, kidneys, and intestines did not show pathological changes. The external appearance of these organs was comparable with those of healthy animals. The mass of the studied organs and the size of the esophagus and intestines were normal.

The results suggest that this drug at doses of 150, 500, and 1000 mg/kg is nontoxic and can be used to study antitumor activity. This is an important property for practical application because most drugs with anticancer activity are highly toxic. This limits their use [10].

Then the experimental tumor strain small-intestine adenocarcinoma (AKATON) was passaged.

Experimental animals were divided into three test groups. Group I received sp-2 *per os* for 10 d with a single dose of 150 mg/kg each day. Group II received sp-2 subcutaneous (s.c.) injections 10 times daily at a dose of 5 mg/kg. Group III was the control with daily administration *per os* and subcutaneously of physiological saline.

Administration to the animals of sp-2 at a dose of 150 mg/kg *per os* (group I) and 5 mg/kg s.c. (group II) decreased significantly both the tumor tissue mass and its volume. Group II, where the animals received sp-2 as s.c. injections, showed the greatest inhibition of tumor growth by 69.91% of the tumor mass and 75.21% of the tumor volume. The inhibition of tumor growth in group I, where the animals received sp-2 *per os*, was 46.18% by mass and 46.95% by volume.

However, measurements of tumor mass and volume are not always adequate to describe the progression or regression of tumor tissue. This is due to the fact that a high degree of exudation, neutrophil invasion, areas of necrotic destruction, or a high differentiation of cell components that accompany malignant transformation of tissue can change the tumor mass in one direction or the other. A more objective criterion of the proliferative activity of tumor cells is a count of the number of mitoses in the tumor tissue, its mitotic index (MI). Another parameter that describes death processes of tumor cells is the apoptotic index (AI) [11].

The number of dividing tumor cells in group I decreased by 66.26%; group II, 70.84% compared with the control group. The number of cells found in apoptosis in the test groups was twice that of cancer cells undergoing division.

The ratio of AI to MI (AI/MI) demonstrated the degree of regression or progression of the tumor tissue. If AI/MI was greater than unity, tumor regression was observed. The AI/MI ratio for group I was 1.75; group II, 2.07; the control, 0.81.

The use of any therapeutic substance that exhibits a cytotoxic effect against tumor cells creates a risk of reducing the proliferation of normal highly proliferative cells. Some of the primary targets of such a side effect are bone marrow cells, disruption of the proliferation of which causes serious negative consequences for hemopoesis and effective operation of the immune system. We studied the inhibitory action of sp-2 on the proliferation of bone marrow cells during studies of its antitumor properties using experimental cancerogenesis in the AKATON tumor model. Protein sp-2 did not inhibit proliferation of bone marrow cells. Therefore, it can be concluded that sp-2 would not have negative effects on normal highly proliferative cells while retaining its antitumor activity.

Analogous results were obtained for B-16 melanoma strains and large-intestine adenocarcinoma (AKATOL) [12, 13]. According to the literature, protein isolates from bean cultures are helpful for various tumorous diseases (adenocarcinoma, angiosarcoma, melanoma, etc.) of the liver, lungs, stomach, intestines, nasopharynx, and connective tissue in mice, rats, and hamsters [14, 15].

A protein complex (protein sp-2) was isolated from soy meal and characterized. The MW (30 kDa) and isoelectric points of the protein components of the most active fraction were determined. It was found that sp-2 at a dose of 100 μg/mL suppressed by 64% incorporation of ^{14}C-thymidine marker, inhibited cancer tissue growth by greater than 60%, and did not inhibit proliferation of bone marrow cells.

EXPERIMENTAL

Isolation of Protein and Column Chromatography. The method for isolating sp-2 included extraction by Tris·HCl buffer (0.05M, pH 7.4). The resulting suspension was filtered to remove insoluble matter. Purification by chromatography used the literature method [16] in Tris·HCl buffer (0.05M, pH 7.4) over a column of Sephadex G-75 (80 × 2.5) at flow rate 20 mL/h. Fractions of 5 mL were collected. Purification by chromatography over AcA34 ultragel (75 × 2.5) used NaCl buffer (0.5M, pH 7.4) at flow rate 20 mL/h. Fractions of 4 mL were collected. Dialysis was carried out against distilled water. The protein solution was lyophilized.

Cytotoxicity Test by ^{14}C-thymidine Incorporation Radiometric Method. The cytotoxic effect of the drugs on growing cells in the logarithmic growth phase during the whole cell cycle was estimated by a ^{14}C-thymidine incorporation radiometric method. The method consisted essentially of KML cells innoculated (40,000 per mL) in RPMI-1640 growth medium (3 mL) in tubes that were treated after 24 h with a compound at a dose of 100 μg/mL. The compounds and cells were in contact for 24 h in order to include the whole cell cycle. After this, ^{14}C-thymidine (10 μCi/tube) was added for 1 h. Then cells were removed with versene from the glass surface, transferred onto GFC filters, and rinsed to remove non-bonded marker. Dried filters were placed into scintillation cocktail (SZh 106) and the amount of radioactivity was determined in a β-counter [17].

Determination of Protein Content in Fraction. Protein was determined by the Lowry method [18].

Electrophoresis of Proteins. The homogeneity of the drug was checked by electrophoresis as before [19].

Isofocusing of proteins was carried out by the Osterman method with certain modifications in PAAG (7.5%, T = 7.5, C = 3.0) in the presence of urea (8M) [20].

Determination of Amino-acid Composition of Protein Fraction. The amino-acid composition of the active protein fraction was determined after acid hydrolysis by HCl (5.7N) at 110°C for 24 h. The hydrolysate was analyzed on a T 339 amino-acid analyzer (Czech Rep.).

Determination of *N*-terminus Amino Acids. *N*-Terminus amino acids were determined by the literature method [21].

Determination of Antitrypsin Activity of Sp-2. Inhibition of trypsin (Fluka, USA) was carried out in a thermostat at 37°C for 10 min using casein hydrolysis (Sigma, USA). The reaction mixture (3.0 mL) consisted of casein (300 μg) and trypsin (3 μg) in a 100:1 ratio with sp-2 (3 μg) in the experimental version [22]. Inhibition of trypsin activity (ITA) was expressed in percent relative to a control (without inhibition) using the formula

$$\mathrm{ITA\%} = 100 - \frac{\mathrm{A_{280}expt} \times 100}{\mathrm{A_{280}control}}$$

Antitumor Activity of Sp-2 in Experimental Cancerogenesis. A tumor strain was passaged according to published instructions [23]. Animals were weighed before injection and after the experiment. The mass of the animals at the end of the experiment was determined by subtracting the tumor mass.

Tumors were measured (in three projections) after sacrificing the animals. Tumor mass and average volume at the end of the experiment were found using the formula [24]

$$V_{av} = \left\{\frac{\pi}{6}\right\} ABC ,$$

where A, B, and C are the tumor length, width, and height; V_{av}, the average tumor volume (cm^3).

Animals were sacrificed under ether anesthesia at least 12 d after the last injection of the studied compounds. The control was a group of animals injected with the solutions used to dissolve the tested compounds, i.e., physiological saline.

Tumor (1 cm^3) for histological examination was taken from mice after the experiment was complete. A portion was preserved in normal formalin (10%). Then, tissue was set in paraffin. Slices (3–5 μm) were prepared for histological samples, dying them with a hematoxylin + eosin mixture. Cells in apoptosis according to the following features were counted in the samples under a microscope. The features were blebbing of the plasmatic membrane, condensation of nuclear chromatin along the nucleus periphery, shrinkage (pycnosis), formation of high-molecular-weight DNA fragments (karyorrhexis), splitting of DNA into oligonucleosomal fragments (staircase type), compaction of cellular organelles, decreased cytoplasm volume, bubbly shape of the cellular membrane, budding of cell fragments with formation of discrete apoptotic bodies surrounded by a membrane and containing compacted remains of organelles and the nucleus [25]. The amount of apoptotic cells was expressed in percent relative to the total amount of counted cells.

The MI of tumor tissue was also determined in the histological samples. For this, the number of cells in division was counted under a microscope. The MI was calculated from this using the formula

$$\mathrm{MI} = \frac{\text{The number of cels in division}}{1000} 100\% .$$

The MI was calculated in 20–25 parts of the tumor. In total, 1000 cells per animal should be counted [26].

The proliferative activity of bone marrow cells was determined using a direct method of hourly cultivation of cells for Fitzgerald chromosome analysis [27].

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