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(71) Applicant *(for all designated States except US)*: **INSTYTUT MEDYCYNY DOŚWIADCZALNEJ I KLINICZNEJ PAN** [PL/PL]; ul. Pawińskiego 5, PL-02-106 Warszawa (PL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LIPKOWSKI, Andrzej** [PL/PL]; ul. Symfonii 47/4, PL-02-786 Warszawa (PL). **MARKOWICZ, Sergiusz** [PL/PL]; ul. Strzeleckiego 2/ 37, PL-02-776 Warszawa (PL).

(74) Agent: **WITEK, Rafał**; WTS Patent Attorneys, Witek, Sniezko & Partners, Weigla 12, PL-53-114 Wrocław (PL).

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(54) Title: PEPTIDE PREPARATIONS AND PEPTIDES WITH ANTITUMOUR ACTIVITY

(57) Abstract: The subject of the present invention are peptide preparations obtained via the enzymatic digestion of hair, wool, bristles, animal fur and individual peptides with sequences corresponding to individual components of a peptide preparation with antitumour activity, for use in the treatment of tumours or oncological prophylaxis as basal components or components of compositions of substances for treating tumours or components of substances used in oncological prophylaxis.

Peptide preparations and peptides with antitumour activity

The subject of the present invention are peptide preparations produced as a result of the enzymatic digestion of hair, wool, bristles, animal fur and individual peptides with sequences corresponding to individual components of peptide preparations with antitumour activity for use in the treatment of tumours or oncological prophylaxis as basal components or components of compositions of substances for treating tumours or components of substances used in oncological prophylaxis.

It is commonly accepted that hair, fur and bristles constitute natural, physical protection for an organism against mechanical trauma and thermal protection against excessive heat gain/loss. However, despite the great resistance against natural factors that degrade hair, fur or bristles, there exists the possibility of degrading their component proteins by the enzymes of bacteria and fungi that colonize the skin as well as by endogenous enzymes secreted in sweat. It is also observed that animals lick and swallow their fur, which may be partially degraded in the digestive tract, thereby secreting appropriate peptide fragments. Attempts have been made to seek the biological role of peptides constituting hair, fur or bristles. Patent WO 03/064449 describes and claims the use of active peptides from hair or wool in wound healing. Our studies on the conversion of hair or fur or bristles have made it possible to formulate an original method of converting using chemical activation and then pepsin digestion. This method was described and claimed in 1995 by B. Baranowska, A.W. Lipkowski, E. Marczak, I. Makulec, H. Rybak, J. Pastuszak, in "*A method of activating keratinous substances by*

enzymatic hydrolysis ", Polish Patent Nr 179342.

While studying the biological activity of a preparation produced through the enzymatic digestion of hair or wool or animal fur substrates initially activated by alkali activity, it unexpectedly turned out that these preparations exhibit significant antitumour activity against tumours of various aetiology. Further analysis of the peptides constituting the preparations showed that individual component peptides also demonstrate antitumour activity. For this reason, peptide preparations obtained through the appropriate digestion of hair or wool or animal fur may be used as active ingredients in the treatment or prophylaxis of tumours or that constitute components of substances for treatment or prophylaxis.

Examples of activities are given to better illustrate the present invention, the antitumour activities of the resulting preparations or individual component peptides. The scope of the present invention, however, should not be limited solely to the wording of the following examples.

Example 1.

Hair from a 60-year old man was activated and hydrolysed with pepsin according to the method described in A.W. Lipkowski, B. Gajkowska, A. Grabowska, K. Kurzepa, "Keratin-associated protein micromaterials for medical and cosmetic applications.", Polimery, Vol. 54, p. 386-388, 2009.

Hair (20 g) has been stirred in 0.1 N sodium hydroxide in room temperature for 2 hours. Then hair was filtered off and washed twice with water. Residue has been suspended in water, temperature has been adjusted to 40°C. pH has been adjusted with 10% hydrochloric acid to 1.6. Pepsin (5 mg) has been added and reaction mixture has been stirred at 40°C. The pH has been controlled and adjusted to pH 1.6-1.9 with hydrochloric acid. After 5 hours solid residue has been washed out, and filtrate has been heated to 80°C

and after 3 minutes, frozen down to -20°C temperature, and lyophilised. The residual powder has been used for biological assays.

The portion dissolved as a result of pepsin digestion was yielding the preparation for further experiments. An analysis of the peptides in the preparation demonstrated the presence of peptides with the sequences given in the table and larger peptide with sequences given in the table.

Table.

Item.	Identified short peptide sequences in the preparation obtained from human hair (amino-acid residues given in the standard single-letter code)
1	AEIRSDL
2	VVQIDNAKL
3	LVVQIDNAKL
4	NKQVVSSSEQL
5	LNKQVVSSSEQL
6	RQLVESDINGL
7	TESEARYSSQL
8	VVQIDNAKLAADDF
9	NRVLNETRSQYEAL
10	NKQVVSSSEQLQSYQAEIIELR
11	NKQVVSSSEQLQSYQAEIIEL
12	LNKQVVSSSEQLQSYQAEIIEL
13	VVNIDNAKL
14	NKQVVSSSEQL
15	LNKQVVSSSEQL
16	NVEVDTAPTVDL
17	VVNIDNAKLASDDF
18	VVEIDNAKL
19	IQEIDF
20	IDKVRF
21	LEQQNKL
22	ASELNHVQEVL
23	NQQVVSSSEQL
24	NQQVVSSSEQL
25	RQLVESDINGL
26	VVEIDNAKLAADDF
27	IVQIDNAKLAADDF
28	TVIFDTGSSNL
29	GILGPVIKAEVGDTL
30	IQEIDF
31	IDKVRF
32	VVQIDNAKL

33	AEIRSDL
34	AEIRSDLE
35	AEIRSDL
36	VVQIDNAKLA
37	VVQIDNAKL
38	NETRSQYEAL
39	NKQVVSSSEQLQ
40	NKQVVSSSEQL
41	LNKQVVSSSEQL
42	RQLVESDINGL
43	VVQIDNAKLAADDF
44	SQVQSLITNVESQL
45	SQVQSLITNVESQLA
46	NRVLNETRSQYEALV
47	NRVLNETRSQYEAL
48	LNRVLNETRSQYEAL
49	NKQVVSSSEQLQSYQAEIIEL
50	NKQVVSSSEQLQSYQAEIIELR
51	AEIRSDLE
52	AEIRSDL
53	LAEIRSDL
54	VVQIDNAKLA
55	VVQIDNAKL
56	NETRSQYEALV
57	NKQVVSSSEQLQ
58	NKQVVSSSEQL
59	LNKQVVSSSEQL
60	RQLVESDINGL
61	SQVQRLITNVESQLA
62	SQVQRLITNVESQL
63	NQVLNETRSQYEALV
64	LNQVLNETRSQYEAL
65	NQVLNETRSQYEAL
66	NKQVVSSSEQLQSYQAEIIELR
67	NKQVVSSSEQLQSYQAEIIEL
68	VVNIDNAKLA
69	VVNIDNAKL
70	NETRSQYEALV
71	NKQVVSSSEQLQ
72	NKQVVSSSEQL
73	LNKQVVSSSEQL
74	VVNIDNAKLASDDF
75	SQVQSLITNVESQLA
76	SQVQSLITNVESQL
77	NQVLNETRSQYEALV
78	LNQVLNETRSQYEAL
79	NQVLNETRSQYEAL
80	LGRVTIAQGGVL

81	PKKTESHHKAKGK
82	IDKVRF
83	LEQQNKL
84	RATAENEF
85	IREYQEVMNSKLGL
86	QNQLEKLG
87	LQNQLEKL
88	QNQLEKL
89	LGKVTIAQGGVLP
90	PKKTESHHKAKGK
91	YRPWGSGSGFG
92	YRPWGSGSGF
93	ERIAGEASRL
94	AKHAVSEGTKAVTKYTSSK
95	DRANNQVGLAPVA
96	FDRANNQVGLAPVA
97	IGGITGPIAKL
98	MEARGPGELC
99	MEARGPGELC
100	IERIPEL
101	TVIFDTGSSNL
102	LIPWVQKPIIF
103	GKEPLGPAL
104	IVNTNVPRASVPDGF
105	MALPVTAL
106	KVGINYQPPTVVPGGDL
107	IDTSRHYLPVKIIL
108	LGRIPSAVGYQPTL
109	VINGNPITIF
110	MKSCGVSL

The preparation was added at 0.1% concentration into the culture media of tumour cells. A parallel control culture was maintained under identical conditions, but without added preparation. The cultures were maintained over a standardised number of days, under typical conditions. The culture medium was exchanged daily both in the control and experimental cultures. In the experimental culture, the new medium always contained 0.1% of the preparation. After seven days, the number of cells was evaluated in the control and experimental cultures. It turned out that in the case of human melanoma cells, in the control culture the number of cells grew 20-fold over 7 days. In the experimental culture containing 0.1% of the preparation, however, the number of cells remained at the initial

level from the outset of the culture. In the case of a 4 day culture of urinary bladder tumour cells, a 0.1% addition of preparation in the culture inhibited the growth of the cells to 70% of the control population. In the case of human lymphoma in a 4 day culture, a 0.1% addition of preparation caused a decrease of proliferation to 35% of that of control cells.

Example 2.

The hair of a 28-year old woman was activated and hydrolysed with pepsin according to the method described in Example I. The portion dissolved as a result of pepsin digestion was lyophilised yielding the preparation for further experiments. The preparation was added at 0.1% concentration into the culture media of tumour cells. A parallel control culture was maintained under identical conditions, but without added preparation. The cultures were maintained over a standardised number of days, under typical conditions. The culture medium was exchanged daily both in the control and experimental cultures. In the experimental culture, the new medium always contained 0.1% of the preparation. After seven days, the number of cells was evaluated in the control and experimental cultures.

It turned out that in the case of human melanoma cells, in the control culture the number of cells grew 20-fold over 7 days. In the experimental culture containing 0.1% of the preparation, however, the number of cells remained at the initial level from the outset of the culture. In the case of a 4 day culture of urinary bladder tumour cells, a 0.1% addition of preparation in the culture inhibited the growth of the cells to 50% of the control population. In the case of human lymphoma in a 4 day culture, a 0.1% addition of preparation completely inhibited the proliferation of the cells.

Example 3

Murine bristles were activated and pepsin hydrolysed according to the method described in Example I. The portion dissolved as a result of pepsin digestion was lyophilised yielding

the preparation for further experiments. The preparation was added at 0.1% concentration into the culture media of tumour cells. A parallel control culture was maintained under identical conditions, but without added preparation. The cultures were maintained over a standardised number of days, under typical conditions. The culture medium was exchanged daily both in the control and experimental cultures. In the experimental culture, the new medium always contained 0.1% of the preparation. After four days, the number of cells was evaluated in the control and experimental cultures. It turned out that in the case of murine melanoma cells, the addition of the preparation completely inhibited the proliferation of the cells.

Example 4

From the list of peptides in the hydrolysate, we arbitrarily selected the peptide sequences 91 (YRPWGSGSGFG) and 92 (YRPWGSGSGF). These peptides were synthesized using the standard Fmoc procedure on a solid phase beginning with appropriate Wanga resin derivatives, Fmoc-Gly-Wang or Fmoc-Phe-Wang. To deprotect the protective group we used 2% piperidine w dimethylformamide (DMF). To attach the subsequent amino-acid we used a 1.5 excess of the appropriate Fmoc-amino-acid in the presence of a 1.5 -fold excess of HATU (O-azobenzotriazol-1yl)-1,1,3,3-tetramethyluronium hexa fluorophosphate)). After synthesizing the peptide with a sequence corresponding to that of peptides 91 or 92, we treated the resin with 99.5% trifluoroacetic acid for 3 minutes. The acid solution was filtered off and rinsed with two small volumes of acid. The combined filtrates were supplemented with a 5-fold volume of an ethyl-ether and hexane mixture (2:1). The precipitate was drained off and washed with the ethyl ether and hexane mixture. The precipitate was dried and purified chromatographically using high-performance preparative chromatography in a gradient of solution A - 0.1% HCl and solution B-methanol. The resulting pure peptides in the form of hydrochlorides were used in biological tests.

The synthetic peptides were examined for their effect on the proliferation of melanoma cells of the line MeW 155. The culture was maintained under standard conditions for 3 days with 4000 cells per well of a 96-well culture plate. After three days, the numbers of cells in the control group increased to 120 000. It turned out that after 3 days of culture, peptide 91 at a concentration of 0.004 mM decreased cell numbers to 55% of the control, and a 0.02 mM concentration decreased the cells to 35%, whereas 0.1 mM was toxic to MeW 155 cells. Peptide 92 at 0.004 mM decreased the number of tumour cells to 65% of the control, a 0.02 mMol concentration decreased the number of cells to 49%, and a concentration of 0.1 mM was toxic to melanoma cells.

PK/1420/RW

Claims

1. A peptide preparation with antitumour activity **characterised in that** it contains an enzymatic hydrolysate of proteins from human hair or bristles or animal fur.
2. A peptide with antitumour activity selected from a group encompassing the peptides with the following sequences

1	AEIRSDL
2	VVQIDNAKL
3	LVVQIDNAKL
4	NKQVVSSSEQL
5	LNKQVVSSSEQL
6	RQLVESDINGL
7	TESEARYSSQL
8	VVQIDNAKLAADDF
9	NRVLNETRSQYEAL
10	NKQVVSSSEQLQSYQAEIIELR
11	NKQVVSSSEQLQSYQAEIIEL
12	LNKQVVSSSEQLQSYQAEIIEL
13	VVNIDNAKL
14	NKQVVSSSEQL
15	LNKQVVSSSEQL
16	NVEVDTAPTVDL
17	VVNIDNAKLASDDF
18	VVEIDNAKL
19	IQEIDF
20	IDKVRF
21	LEQQNKL
22	ASELNHVQEVL
23	NQQVVSSSEQL
24	NQQVVSSSEQL
25	RQLVESDINGL
26	VVEIDNAKLAADDF
27	IVQIDNAKLAADDF
28	TVIFDTGSSNL
29	GILGPVIKAEVGDTL
30	IQEIDF
31	IDKVRF
32	VVQIDNAKL
33	AEIRSDL
34	AEIRSDLE
35	AEIRSDL

36	VVQIDNAKLA
37	VVQIDNAKL
38	NETRSQYEAL
39	NKQVVSSSEQLQ
40	NKQVVSSSEQL
41	LNKQVVSSSEQL
42	RQLVESDINGL
43	VVQIDNAKLAADDF
44	SQVQSLITNVESQL
45	SQVQSLITNVESQLA
46	NRVLNETRSQYEALV
47	NRVLNETRSQYEAL
48	LNRVLNETRSQYEAL
49	NKQVVSSSEQLQSYQAEIIEL
50	NKQVVSSSEQLQSYQAEIIELR
51	AEIRSDLE
52	AEIRSDL
53	LAEIRSDL
54	VVQIDNAKLA
55	VVQIDNAKL
56	NETRSQYEALV
57	NKQVVSSSEQLQ
58	NKQVVSSSEQL
59	LNKQVVSSSEQL
60	RQLVESDINGL
61	SQVQRLITNVESQLA
62	SQVQRLITNVESQL
63	NQVLNETRSQYEALV
64	LNQVLNETRSQYEAL
65	NQVLNETRSQYEAL
66	NKQVVSSSEQLQSYQAEIIELR
67	NKQVVSSSEQLQSYQAEIIEL
68	VVNIDNAKLA
69	VVNIDNAKL
70	NETRSQYEALV
71	NKQVVSSSEQLQ
72	NKQVVSSSEQL
73	LNKQVVSSSEQL
74	VVNIDNAKLASDDF
75	SQVQSLITNVESQLA
76	SQVQSLITNVESQL
77	NQVLNETRSQYEALV
78	LNQVLNETRSQYEAL
79	NQVLNETRSQYEAL
80	LGRVTIAQGGVL
81	PKKTESHHKAKGK
82	IDKVRF
83	LEQQNKL

84	RATAENEF
85	IREYQEVMNSKLGL
86	QNQLEKLG
87	LQNQLEKL
88	QNQLEKL
89	LGKVTIAQGGVLP
90	PKKTESHHKAKGK
91	YRPWGSGSGFG
92	YRPWGSGSGF
93	ERIAGEASRL
94	AKHAVSEGTKAVTKYTSSK
95	DRANNQVGLAPVA
96	FDRANNQVGLAPVA
97	IGGITGPIAKL
98	MEARGPGELC
99	MEARGPGELC
100	IERIPEL
101	TVIFDTGSSNL
102	LIPWVQKPIIF
103	GKEPLGPAL
104	IVNTNVPRASVPDGF
105	MALPVTAL
106	KVGINYQPPTVVPGGDL
107	IDTSRHYLPVKIIL
108	LGRIPSAVGYQPTL
109	VINGNPITIF
110	MKSCGVSL

3. A peptide preparation for use in antitumour therapy or prophylaxis **characterised in that** it contains at least one of the peptides defined in Claim 2.
4. Peptide preparation according to Claim 3, **characterised in that** it is in the form of a cream, ointment, cosmetic liquid or oral preparation.
5. A peptide preparation according to Claim 3, **characterised in that** it is a component of an implant or ointment for use in therapy for preventing tumour recurrence.