The washing ethereal fraction, after the solvent had been distilled off, yielded 0.007 g of benzoic acid, and the main ethereal extract gave 0.039 g of karasamine.

Benzoylation of Karasamine. A solution of 0.09 g of karasamine in 3 ml of pyridine was treated with 0.05 g of benzoyl chloride and the mixture was kept at room temperature for 4 h. The excess of pyridine was distilled off in a rotary evaporator, the residue was dissolved in water, and the solution was made alkaline with sodium carbonate and was extracted with ether. After the solvent had been distilled off, with the aid of acetone 0.053 g of 1-O-benzoylkarasamine was isolated.

SUMMARY

The alkaloids of the epigeal part of Aconitum karakolicum Rapaics. have been studied, and the known alkaloids phenyl-β-naphthylamine, karakoline, neoline, delsoline, monticamine, songorine, napelline, acetylnapelline, napelline N-oxide, and isoboldine and two new alkaloids – karasamine and 1-O-benzoylkarasamine have been isolated.

On the basis of a spectral characteristics and chemical transformations, structures have been proposed for karasamine and 1-O-benzoylkarasamine.

LITERATURE CITED

1. M. N. Sultankhodzhaev and M. M. Tadzhibaev, Khim. Prir. Soedin., 406 (1976).
2. M. N. Sultankhodzhaev, L. V. Beshitaishvili, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 681 (1976).
3. M. N. Sultankhodzhaev, L. V. Beshitaishvili, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 479 (1978).
4. M. N. Sultankhodzhaev, L. V. Beshitaishvili, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 826 (1979).
5. M. N. Sultankhodzhaev, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 660 (1982).
6. M. S. Yunusov, Ya. V. Rashkes, V. A. Tel'nov, and S. Yu. Yunusov, Khim. Prir. Soedin., 515 (1969).
7. S. W. Pelletier, L. H. Keith, and P. S. Parthasarathy, J. Am. Chem. Soc., 89, 4146 (1967).
8. M. N. Sultankhodzhaev, M. S. Yunusov, and S. Yu. Yunusov, Khim. Prir. Soedin., 199 (1973).

ISOLATION AND STUDY OF THE PROPERTIES OF AN INTERFERON-LIKE INHIBITOR OF VIRUSES FROM NORMAL HUMAN BLOOD SERUM

G. T. Mavlanov, S. A. Auelbekov, and Kh. A. Aslanov

UDC 577.112.083:578.282

A protein with a molecular weight of 17,400 daltons and an isoelectric point at pH 4.9 has been isolated from the blood serum of healthy donors by successive ion-exchange chromatography of QAE-Sephadex A-50, affinity chromatography on DNA-cellulose, and polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate. The protein isolated, like interferon, suppresses the development of the cytopathogenic action of the viruses of vesicular stomatitis and murine ecephalomyocarditis in cultures of human cells of the L-41 and M-19 lines. The amino acid composition of the protein isolated differs from those of various fractions of human interferons.

The development of the state of resistance of a higher organism to viral infection is brought about not only by the induction of interferon but also by the presence (or induction) of other nonspecific viral inhibitors [1]. The detection and partial characterization of

V. I. Lenin Tashkent State University. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 210-215, March-April, 1966. Original article submitted July 11, 1985.

0009-3130/86/2202-0195 $12.50

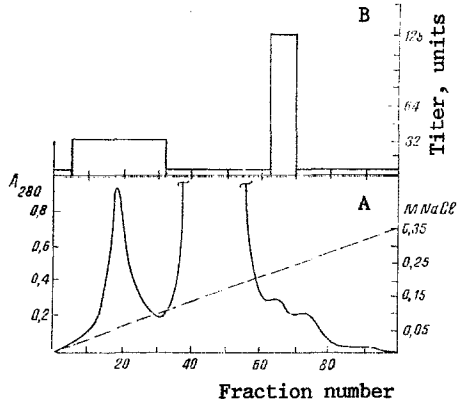

Fig. 1

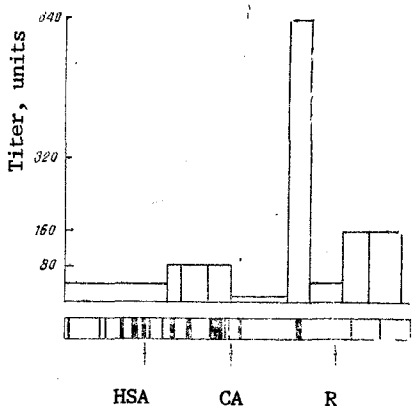

Fig. 2

Fig. 1. Ion-exchange chromatography of the proteins of human blood serum on QAE-Sephadex A-50: A) protein-elution profile; continuous line, UV absorption of the fraction; dashed line, concentration gradient of sodium chloride; B) titers of antiviral activity in M-19 cells.

Fig. 2. Electrophesis of a fraction of DNA-binding serum proteins with antiviral activity in 15% polyacrylamide gel in the presence of NaDDS. At the bottom: stained gel, the columns representing titers in L-41 cells. The arrows show the positions of the marker proteins: HSA) human serum albumin (68,000); CA) carboanhydrase (30,000); R) RNase A (13,700).

such inhibitors has been the subject of prior investigations [2-4]. The presence of inhibitors of this category was revealed from a decrease in the infection titer of the virus and from the suppression of a viral function – for example, hemagglutination. There is no information in the literature on the isolation of homogeneous inhibitors and their chemical characterization. In some investigations, any inhibitor detected by the authors is characterized as "trypsin-sensitive molecules," "a lipoprotein," or "a sulphopolysaccharide." Inhibitors of viruses have also been detected in human serum: of the coronavirus OC-43 [5], of vesicular stomatitis virus [6], and of Friend's leukemia virus [7]. However, human serum in the norm does not exhibit antiviral activity in an interferon test system [8]. On the other hand, substances have been detected in sera and other tissue fluids that suppress the action of interferon [9-11]. In view of this, we have made an attempt to fractionate serum with the aim of detecting antiviral activity on the assumption that as the result of fractionation it will be possible to reveal an antiviral activity "freed" from antagonists.

In this work we used native serum from healthy donors. To eliminate antibodies against the test viruses that may have been present, the immunoglobulins were precipitated from the serum by salting-out with ammonium sulfate at 33% saturation; the albumin fraction of the serum proteins, just like the native serum, exhibited no antiviral activity. The albumin fraction was then fractionated by ion-exchange chromatography on the anion-exchanger QAE-Sephadex. When the bound proteins were eluted with a linear concentration gradient of sodium chloride, four fractions were obtained. The testing of the antiviral activities of these fractions in the interferon test system showed that two of them possessed such activity (Fig. 1).

We may note that the elution profile of antiviral activity of the serum protein given in Fig. 1b has a similarity with the elution profile of common leukocytic inferon for this ion-exchanger [12]. In view of this circumstance, in the subsequent purification of the antiviral factor of the serum we turned to the methods of purifying human interferons that have been described in [12-15]. In particular, in [12] to purify human leukocytic interferon we used affinity chromatography on DNA-cellulose, and this was selected as the next stage of fractionation. Because of its high specific and total antiviral activity, for affinity chromatography on DNA-cellulose we selected the material that was eluted from the QAE-Sephadex in fractions 63-70. When a protein fraction possessing antiviral activity was deposited on the affinity column, it was bound completely to the sorbent and could be eluted with 0.05 M NaCl:

Fraction	Volume, ml	Protein, mg	Titer in L-41 cells	Titer, % of the initial activity
Eluate from ion exchanger, fractions 63-70 (Fig. 1)	50	17.0	1280	100
Not bound to the DNA cellulose	220	14.9	20	6.8
0.05 M NaCl eluate	50	0.9	640	50
0.5 M NaCl eluate	50	0.49	160	12.5

Since the bulk of the antiviral activity was eluted from the DNA cellulose by 0.05 M NaCl, we subsequently collected only this fraction.

To determine the molecular weight of the antiviral factor, the eluate from the DNA-cellulose was subjected to electrophoresis in 15% polyacrylamide gel in the presence of sodium dodecyl sulfate (NaDDS). Part of the gel was stained with Coomassie Bright Blue R-250 and sections of another part were cut out at the positions corresponding to the colored bands and the protein was extracted in order to determine its antiviral activity. The results are given in Fig. 2.

As can be seen from Fig. 2, the bulk of the antiviral activity was revealed in the band corresponding to a molecular weight of 17,400 daltons, this value of the molecular weight of the inhibitor from serum being close to the molecular weight of leukocytic interferon [13].

The homogeneous inhibitor isolated by comparative electrophoresis exhibited 10^5 international units of interferon antiviral activity per 1 mg of protein in L-41 cells.

As is well known, the interferons are heterogeneous both with respect to charge and with respect to molecular dimensions. At the present time, from the various forms of interferons homogeneous components have been isolated and their amino acid compositions and terminal amino acid residues and also, for some components, the primary structures of parts of the molecule have been determined. An analysis of the available information on the amino acid compositions and N-terminal sequences (20-30 residues out of 165) shows that interferons of the same species origin have different structures. From the positions of certain amino acids and even of groups of 2-4 amino acids human lymphoblastoid interferon resembles the structure of murine "C" interferon, while there are practically no such coincidences between lymphoblastoid and fibroblast interferons – out of 15 amino acids from the N-end only in position 9 is there the same residue in each case – leucine [17]. Thus, it is still impossible, from the available information on the chemistry of the interferons, to find a link between antiviral activity and structure. The fact that they have different structures possibly indicates different molecular mechanisms of their action.

The homogeneous viral inhibitor was subjected to amino acid analysis for comparison with human interferons of various types: keukocytic [13] (molecular weight 18,000; N-terminal residue cysteine); lymphoblastoid [14] (molecular weight 18,800; N-terminal residue serine); and fibroblast [15] (molecular weight 20,000; N-terminal residue methionine). The terminal amino acid residue of the serum viral inhibitor is cysteine. Information on the amino acid compositions of the viral inhibitor and of the interferons is given in Table 1.

As can be seen from Table 1, the viral inhibitor that we have isolated from normal serum differs with respect to its amino acid composition from three types of human interferons for which the amino acid composition is known.

The isoelectric point of the inhibitor was determined by electrofocusing in 5% polyacrylamide gel with Ampholines and proved to be 4.9. It is known from the literature that human interferons are heterogeneous with respect to charge and have isoelectric points from pH 3.5 to 10. The heterogeneity of leukocytic interferon can be eliminated by removing the carbohydrate moiety of the molecules [23]. The charge of leukocytic interferon after deglycosylation proved to be approximately pH 5.7.

Thus, from normal human blood serum a homogeneous protein has been isolated which exhibits antiviral activity in a virus-cell system. In this interferon test-system, direct contact of the inhibitor with the test virus is excluded and the antiviral action is effected indirectly through the metabolism of the target cell [18]. The facts given above permit the protein isolated to be called an "interferon-like viral inhibitor." In contrast to the interferon synthesized inducibly in response to the action of a virus of an inductor, we have

TABLE 1. Amino Acid Compositions of Various Human Interferons and the Serum Interferon-like Viral Inhibitor

Amino acid	No. of residues in the molecule			
	Serum inhibitor*	Leukocytic interferon [13]	Lymphoblastoid interferon [14]	Fibroblast interferon [15]
Asp	16,8	15,2	14,9	18,9
Thr	7,0	7,5	8,0	6,8
Ser	9,8	8,0	10,7	10,5
Glu	18,2	24,0	27,3	27,0
Pro	7,0	6,3	10,9	2,7
Gly	16,8	5,5	10,7	7,8
Ala	7,0	8,2	11,0	10,0
Cys	5,6	3,3	1,8	1,7
Val	7,0	7,8	7,7	6,0
Met	4,2	3,9	1,2	2,9
Ile	5,6	8,9	6,9	9,0
Leu	11,2	21,8	17,8	20,4
Tyr	5,6	5,1	3,8	7,5
Phe	7,0	9,1	7,1	9,4
His	15,4	3,3	4,4	4,9
Lys	8,4	11,6	10,4	11,6
Arg	4,2	7,3	9,6	10,9
Trp	1,4	0,7	0,6	1,0

* Mean value from three experiments.

isolated this inhibitor from various sera and plasmas of the blood of healthy human donors regardless of blood group with practically the same yield – 3.5 ±0.7 μg from 1 ml. One more difference of the serum proteins from the interferons is in their specific activities: for interferons the specific antiviral activity is $2\text{-}4\cdot10^9$ units per 1 mg, and for the inhibitor that we have isolated it is $1\text{-}2\cdot10^5$ units per 1 mg. However, some fractions of interferons also exhibit a lower activity – 10^4 units per 1 mg..

In the process of fractionation – for example, in prolonged dialyses against solutions of a low ionic strength (less than 10 mM) – a sharp decrease in the antiviral titer of the serum inhibitor was observed. This inactivation was reversible, and it was possible to reactivate the protein by heating it in 1-2% NaDDS for one or two minutes at the temperature of the boiling water bath following by cooling to room temperature. Such reversible inactivation is also known for interferons [19] and is possibly connected with the aggregation of the molecules through noncovalent interactions.

A further deepening of the study of the protein found will undoubtedly give useful information on the nonspecific protective forces of the organism that are present in the norm.

EXPERIMENTAL

Native donor plasma suitable for blood transfusion (100 ml) was diluted twofold with 0.14 M NaCl, EDTA was added to a concentration of 5 mM, and the imunoglobulins were precipitated with ammonium sulfate (kh. ch. ["chemically pure"]). With stirring, 39.6 g of the salt was added to 200 ml of the diluted serum. The precipitate was separated off by centrifugation on a K-24 centrifuge (15,000 g, 15 min). The supernatant, consisting of the albumin fraction, was dialyzed against three changes of 10 volumes each of 10 mM potassium phosphate buffer, pH 8.6, with 1 mM EDTA (KPB) and was deposited in portions each containing 500 mg of protein on a 2 × 10 cm column of QAE-Sephadex A-50 (Sweden). The column was washed with 3 volumes of KPB and the bound proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.35 M created with the aid of an Ultragrad.

Fractions with a volume of 6 ml each were collected, and their UV absorptions were measured on a SF-26 spectrophotometer at 208 nm. The fractions possessing antiviral activity were combined, dialyzed against KPB, and deposited with observation of a protein:DNA ratio of 1:2. The column was washed with five volumes of KPB, and the bound proteins were eluted successively with 0.05 and 0.5 M NaCl in KPB, the fraction exhibiting a high antiviral activity (0.05 N NaCl eluate) being collected, dialyzed against 10 mM Tris-HCl, pH 6.8, and freeze-dried. The chromatographic procedures and the dialysis were performed at +6°C.

The DNA-cellulose was obtained by Litman's method [20]. The solvent used in the experiment contained 50 mg of bovine spleen DNA (Reakhim B).

Analytical electrophoresis in a 0.1 × 12 × 12 cm block of 15% polyacrylamide gel containing 0.1% of NaDDS was performed with the disk buffer system described by Laemmli [16] at a current strength of 25 mA for 3.5 h. Preparative electrophoresis was carried out in a 0.3 × 15 × 15 cm block with thermostating at +10°C using an instrument for vertical electrophoresis (LKB, Sweden). The protein bands were revealed by staining with Coomassie Bright Blue R-250. The protein was eluted from the polyacrylamide gel in 0.1 M sodium phosphate, pH 7.2, containing 0.1% of NaDDS and 1 mM phenylmethanesulfonyl fluoride at room temperature for 12 h. The eluate from the gel was dialyzed against 10 mM Tris-HCl, pH 6.8, and was freeze-dried. The dry extract was treated with cooled methanol to eliminate NaDDS and Coomassie, which do not dialyze. The methanol washing was repeated four times in a ratio of 1 ml of methanol to 1 mg of dry eluate.

The antiviral protein was eluted from the gel to determine its antiviral activity with 0.05 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl and 0.01% of NaDDS. Before titration the eluate was dialyzed against 0.1 M NaCl.

The yield of electrophoretically homogeneous protein from 100 ml of the initial serum was 350 μg.

The homogeneous protein was hydrolyzed in sealed tubes with 6 M HCl for 6, 24, and 48 h at 115°C. Analysis of the amino acids present was performed on a Biotronic LC 7000 amino acid analyzer (FRG). Tryptophan was determined after hydrolysis with methanesulfonic acid [22]. The N-terminal residue of the protein was determined by the dansyl method [21].

The isoelectric point was determined by electrofocusing on prepared polyacrylamide plates with Ampholines (LKB 1804 Ampholine PAG plate, pH range 3.5-9.5). After electrofocusing (at an initial voltage of 1200 V, 5.5 h), a band 1.5 cm wide was scraped from the edge of the gel and it was divided into fractions 0.5-cm long. The pH of each fraction of the gel was determined after incubation of the lump in 5 ml of deionized water for 2 h. The section of the gel containing the protein was fixed in 40% methanol containing 10% of acetic acid and was stained with Coomassie Brilliant Blue.

To determine the antiviral activity of the serum protein, monolayers of L-41 and M-19 interferon-sensitive human cells were treated with various dilutions of the protein in cultivation medium, and after 18 h the protein solution was removed and 100 TCDs (tissue cytopathic doses) of murine encephalomyocarditis virus or vesicular stomatitis virus were added. The results were read after the development of the cytodestructive effect in control samples without the inhibitor. Leukocytic interferon with a known reference titer was titrated in parallel. As the interferon (inhibitor) titer was taken the final dilution of the substance at which 50% protection from the cytodestructive action of the test virus was observed.

The antiviral activity in international titers was determined by N. R. Shukhmina (Moscow Scientific-Research Institute of Viral Preparations), and the amino acid compositions by A. Korneev (Institute of Bioorganic Chemistry of the Academy of Sciences of the Uzbek SSR, Tashkent).

SUMMARY

1. It has been shown that normal human plasma and serum contain a protein suppressing the action of cytopathogenic viruses in interferon-sensitive cells of human origin.

2. The molecular weight of the antiviral protein from serum is close to those of human alpha- and beta-interferons, while its amino acid composition differs from those of the interferons.

LITERATURE CITED

1. S. Kumar and S. Baron, Tex. Rep. Biol. Med., 41, No. 395 (1981-1982).
2. S. Baron and L. McKerlie, Infec. Immun., 32, No. 2, 449 (1981).
3. T. K. Hughes, J. E. Blalock, et al., Infec. Immun., 32, No. 2, 454 (1981).
4. Kinjo and R. Yanagawa, Jap. J. Vet. Res., 17, No. 1, 43 (1969).
5. L. L. Suribaldi, N. Seganti, et al., Microbiologica, 2, 121 (1979).
6. L. Thiry, Virol., 87, 384 (1978).
7. M. L. Bendinelli et al., J. Gen. Virol., 22, 207 (1974).

8. F. I. Ershov and A. S. Novokhatskii, Interferon and Its Inductors [in Russian], Moscow (1981).
9. T. G. Rossman and J. Vilček, Arch. Ges. Virusforsch., 31, 18 (1970).
10. M. V. Rytel, Arch. Virol., 48, 103 (1975).
11. T. C. Cesario and J. G. Tilles, Tex. Rep. Biol. Med., 41, 359 (1981-1982).
12. G. T. Mavlanov, S. A. Auelbekov, D. B. Dzhumaev, and Kh. A. Aslamov, Khim. Prir. Soedin., 766 (1983).
13. M. Rubinstein, S. Rubinshtein, et al., Proc. Natl. Acad. Sci. USA, 76, No. 2, 640 (1979).
14. K. C. Zoon, E. Smith, et al., Science, 207, No. 4430, 527 (1980).
15. E. J. Knight and M. V. Hunkapiller, et al., Science, 207, No. 4430, 525 (1980).
16. U. K. Laemmli, Nature (London), 227, No. 5259, 680 (1970).
17. E. J. Knight, in: Interferon 1980, Academic Press, London, Vol. 2 (1980), p. 1.
18. W. E. Stewart, J. Infect. Dis., 142, No. 4, 643 (1980).
19. R. S. Jariwalla et al., Experientia, 36, No. 12, 1390 (1980).
20. R. M. Litman et al., J. Biol. Chem., 243, 6222 (1968).
21. W. R. Gray, Methods Enzymol., 25, 121 (1972).
22. S. Moore, in: Chemistry and Biology of Peptides (ed. G. Meinhofer), Ann Arbor Publishers, Michigan (1972), p. 629.
23. B. W. Stewart II et al., Proc. Natl. Acad. Sci. USA, 74, No. 10, 4200 (1977).

AFFINITY CHROMATOGRAPHY OF CARBOXYLIC PROTEINASES ON A POLYMERIC SORBENT CONTAINING GRAMICIDIN-C AS LIGAND

G. N. Balandina, G. I. Lavrenova, L. D. Uzhinova, E. A. Chekhovskikh, N. A. Platé, and V. M. Stepanov

UDC 577.156.41+577.15.07

A new polymeric sorbent for proteinases has been synthesized by the radical copolymerization of N-vinylpyrrolidone, bis-N^{δ}-acryloylgramicidin C, and N,N'-methylenebisacrylamide. Biospecific chromatography on the new sorbent has enabled an industrial preparation of procine pepsin to be purified by a factor of 2.5. With the aid of the new sorbent, a carboxylic proteinase has been isolated from the industrial preparation Tsellolignorin with a 15-fold purification factor.

Affinity chromatography on sorbents consisting of insoluble supports (Sepharose, Aminosililochrom) covalently linked with the polypeptide antibiotic gramicidin-C is being widely used for the isolation and purification of carboxylic proteinases [1, 2]. However, the low solubility of the antibiotic in water leads to great losses of it during the synthesis of the sorbents, a consequence of which is the low degree of inclusion of the ligand even at high concentrations of gramicidin-C. It is impossible to overcome this difficulty by using organic solvents in the synthesis, since, as a rule, the polysaccharide matrix withstands only relatively low concentrations of them. The maximum inclusion of gramicidin-C in the synthesis of the Sepharose sorbents is 5-8 μmole/ml of sorbent.

The sorbents based on Aminosilochrom [2] are resistant to the action of organic solvents but their synthesis requires a large excess of antibiotic, and the inclusion of the ligand is also low.

To synthesize sorbents with a regulable inclusion and distribution of the ligand it is desirable to use the method of the ternary copolymerization of the monomers forming the matrix and of the ligand modified by the introduction into its molecule of a residue containing a double bond [3]. As the monomers we used N-vinylpyrrolidone, N,N'-methylenebisacrylamide, and bis-N^{δ}-acryloylgramicidin-C. As can be seen from Table 1, a change in the ratio of the monomers led to different inclusions of gramicidin-C in the polymeric matrix.

M. V. Lomonosov State University. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 215-218, March-April, 1986. Original article submitted July 30, 1985.

 0009-3130/86/2202-0200 $12.50