AJPMHS GLOBAL RESEARCH NETWORK

AMERICAN Journal of Pediatric Medicine and Health Sciences
Volume 01, Issue 09, 2023 ISSN (E): 2993-2149

Obtaining Polypeptides and Studying Their Biological Activity

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Abstract: Polypeptides have been obtained for use as carriers in polymer dosage forms containing macro- and microelements. It has been established that the optimal conditions for obtaining water-soluble polypeptides with an average molecular weight of 70,000-75,000 kDa with a maximum yield by hydrolysis of the silkworm cocoon (Bombyx mori) with caustic alkali solutions (NaOH, KOH) are: the concentration of the alkali solution is 0.250 N, the temperature of the system is 373K, the reaction time is 80 minutes. Research has determined that the resulting polypeptides do not have a negative impact on the health of animals, do not cause local irritant effects on the skin and mucous membranes of the eyes, and do not have a cumulative property.

Keywords: polypeptides from Bombyx mori, polymer substrate, hydrolysis, potentiometry, microscopy, electrophoresis, toxicity, local irritant effect, cumulative effect, morphology, biochemistry.

It is known from the scientific literature that sericin and fibroin isolated from the cocoon of Bombyx mori have a certain physiological activity [1, 2], but the conditions for obtaining polypeptides with different functional groups from it by alkaline hydrolysis and their biological activity remain poorly understood. Purposeful studies of the conditions for obtaining water-soluble polypeptides and their physiological activity will serve as the basis for the development of new promising dosage forms based on their polycomplexes with macro- and microelements. The aim of this work was to establish optimal conditions for obtaining polypeptides with a large molecular weight with maximum yield and to determine their pharmacological effect on the condition of animals.

The cocoon of Bombyx mori has a protein nature, therefore, under the influence of caustic alkali solutions, it is easily hydrolyzed first to polypeptides, and with deep hydrolysis to individual amino acids [3]. The influence of the conditions of hydrolysis of the silkworm cocoon with aqueous solutions of NaOH and KOH was studied at temperatures of 323K, 343K, 373K and alkali concentrations from 0.09 n to 0.625 n, as well as the reaction duration up to 210 minutes. The depth of the hydrolysis reaction was estimated indirectly by the method of potentiometry, by determining the magnitude of the change in the pH of the system during the process. It is established that an increase in the concentration, duration of hydrolysis time and temperature of

the system leads to a deepening of the process. It should be noted that during the process at 323K, the optimal state of the reaction product - a homogeneous aqueous solution of polypeptides is not achieved even for a long time (210 minutes), and during the process at 343K, the optimal state of the reaction product is achieved only at a high concentration of alkali (0.625 n) and after 85 minutes of reaction. The goal is achieved when the process is carried out at 373K at a concentration of 0.250 n and after 80 minutes of the reaction.

All reaction products after neutralization were examined using an invented phase contrast microscope "Evos XL Cote" (Germany) - fourfold (4x), tenfold (10x), twenty-fold (20x) and forty-fold (40x) magnification of the lens. An increase in temperature from 323K to 373K has a positive effect on the process of hydrolysis of the silkworm cocoon (Fig. 1), this is explained by an increase in the number of interactions of alkali molecules with proteins or with polypeptides formed during the reaction when the temperature of the system increases. Increasing the concentration of alkali in the solution and the duration of thereaction also leads to a deepening of the hydrolysis reaction, which can be explained by an increase in the number of interactions of alkali molecules with proteins or with polypeptides formed during the reaction, respectively, with an increase in the concentration of the solution or the duration of the reaction.

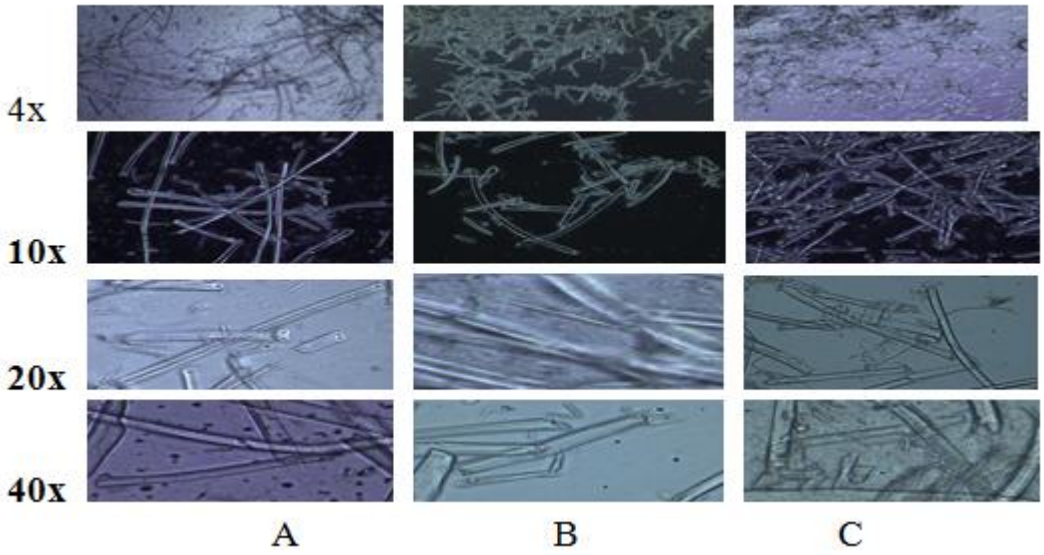


Fig. 1. Microscopic images of the products of hydrolysis of the silkworm cocoon with NaOH solution at a concentration of 0.625 n, the duration of hydrolysis time of 90 min., magnification of the lens by 4, 10, 20, 40 times at temperatures: A – 323K; B - 343K; C – 373K.

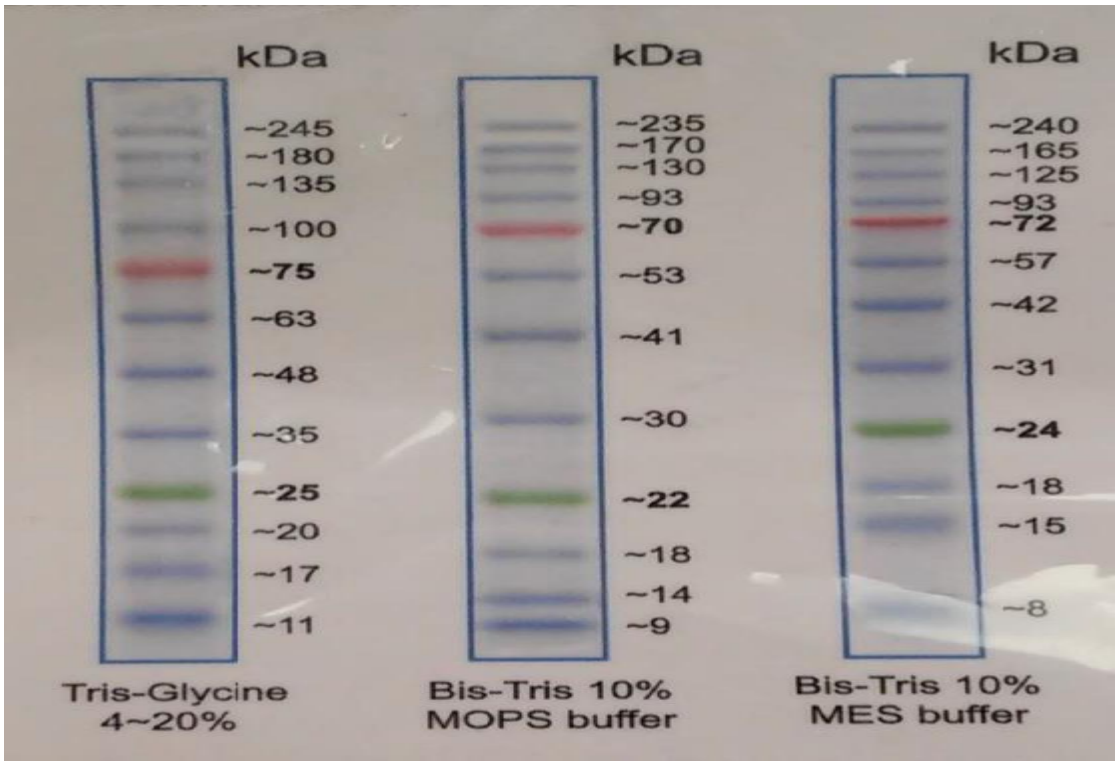
The influence of the nature of alkali on the process of hydrolysis of the silkworm cocoon is determined by the example of studies using KOH and NaOH. Processing of the potentiometry results by mathematical static analysis showed a stronger effect of NaOH on this process. In all subsequent studies, the samples were previously neutralized to pH = 6.8-7.2 by adding the required amount of an aqueous concentrated HCI solution.

The molecular weight of polypeptides obtained during experiments was determined by electrophoresis. The research was carried out on an Electrophoresis Power Supply device (Germany). Sericin with a molecular weight of 220,000 kDa was taken as a standard protein. From Fig. 2 it can be seen that the hydrolysis products are polydisperse, consisting of both low-molecular (≤8,000 kDa) and high-molecular (>240,000 kDa) fractions.

Processing of the obtained results allows us to assert that the optimal conditions for obtaining water–soluble polypeptides with an average molecular weight of 70,000–75,000 kDa by hydrolysis of the Bombyx mori cocoon with alkali solutions (NaOH, KOH) are: the concentration of the alkali solution (C) - 0.250 n, system temperature (T) - 373K, reaction duration (t) – 80 minutes.

As is known [4], the conjunctival test is a very sensitive test and, in some cases, even allows you to detect the reaction of animals to an allergen, with weak allergization and negative skin tests. The experiments were performed on 10 rabbits weighing 2.5-3.0 kg, which were instilled with 0.5 and 5 % solution of the drug in the left eye, 0.1 ml. of distilled water was injected into the second eye (control). The reaction was taken into account after 15 minutes (rapid reaction) and after 24-48 hours (delayed hypersensitivity) and evaluated on the following scale (in points) [5]:

- slight redness of the tear duct;
- redness of the lacrimal duct and sclera in the direction of the cornea;
- redness of the entire conjunctiva and sclera.



A B C

Fig. 2. Photo of markers of mixing of the products of the hydrolysis reaction of the silkworm cocoon with NaOH solution under the conditions: A) C = 0.625 n; T = 373K; t = 60 min.;

B) C = 0.375 n; T = 373K; t = 60 min.; C) C = 0.250 n; T = 373K; t = 80 min.

In addition, the degree of hyperemia, swelling, lacrimation were taken into account. The results of observations showed that the test sample does not cause even a slight redness after 15 minutes, 24 and 48 hours.

The study of the skin-irritant effect was carried out on 15 rats by the introduction of distilled water with an automatic pipette (control), and in the two left 0.5 and 5 % solutions of the sample in a volume of 0.05 ml. The data obtained showed that the test sample in 0.5 and 5 % concentrations does not cause irritation, redness, swelling or other visible skin changes and the effect is estimated at 0 points.

Based on the above data, it can be concluded that polypeptides obtained by hydrolysis of the cocoon Bombyx mori in 0.5 and 5 % concentrations do not have an irritating effect on both the mucous membrane of the eye of rabbits and the skin of rats.

The study of cumulation was carried out using the method of Lima and others [5], allowing to evaluate not only cumulation, but also habituation. Experiments were conducted on 10 mice of

both sexes with an initial body weight of 20±2 g. The drug was administered orally according to the scheme presented in Table 1.

Table 1. Scheme of administration of polypeptides obtained by hydrolysis of cocoon Bombyx mori to mice. The maximum duration of the experiment is 24 = 4 days.

Days of introduction	Number of dead animals / total	Proportionof LD_{50}	LD_{50} >5000 mg/kg
1-4	0/10	0,1	500
5-8	0/10	0,15	750
9-12	0/10	0,22	1100
13-16	0/10	0,34	1700
17-20	0/10	0,50	2500
21-24	0/10	0,75	3750

Based on the results obtained by the method [5], $K_k = LD_{50n} / LD_{50\ 1}$ was calculated, where K_k = cumulation coefficient, LD_{50n} – the average lethal dose with n-fold administration, $LD_{50\ 1}$ – the average lethal dose with a single administration. According to this method, if $K_k > 1$ addiction; $K_k \leq 1$ cumulation. As can be seen from the above data, the test sample does not have a cumulative effect.

Subsequently, the animals (mice) participating in the previous experiment were slaughtered at the end of the observation period, blood was taken to count the shaped elements in the peripheral blood, blood serum was isolated for biochemical studies, macroscopic examination and weighing of internal organs were performed. The condition of the animals was assessed according to the following parameters of peripheral blood: hemoglobin content, the number of erythrocytes, hematocrit, the average volume of erythrocytes in cubic micrometers (MCV), the average hemoglobin content in a single erythrocyte in absolute units (MSN), the average concentration of hemoglobin in the erythrocyte mass, reflecting the degree of saturation of the erythrocyte with hemoglobin (MSNS), reticulocytes, platelets, leukocytes, assessment of liver function - the content of glucose, total protein, alanine and aspartate aminotransferases (AlAT, AsAT) in blood serum.

The results of observations are as follows:

1. During the period of the experiment, the mortality of experimental animals was not observed.
2. There were no clinical signs of intoxication during the period of the experiment.
3. During the autopsy of animals, pathoanatomic changes in organs: liver, kidneys, spleen, heart, lungs, and thymus were not detected.
4. The body weight of the animals taken in the experiment did not significantly differ from the weight of the control group animals (Table 2).
5. The morphological composition of peripheral blood in the experimental animals did not differ from the animals of the control group.

Table 2. Averaged measurements of the mass of animals and internal organs of mice with repeated oral administration of polypeptides obtained by hydrolysis of the cocoon Bombyx mori according to the scheme: M±m; n=10; P>0.05.

Group of animals	issue	after 24 days	liver	kidneys	spleen	heart
Control	20±2,0	22±2,0	1,45±0,1	0,20±0,02	0,14±0,01	0,12±0,01
Test subjects	20±2,0	22±2,1	1,09±0,1	0.23±0,02	0,17±0,01	0,13±0,01

Table 3. Morphological composition of peripheral blood with repeated oral administration of poly-peptides obtained by hydrolysis of cocoon Bombyx moriaccording to the scheme: M±m; n=10; P>0.05

Tests	control	experience
Hemoglobin, g\v	110±6,0	97±9,4
Red blood cells, 1012/l	5,4±0,1	4,8±0,1
Hematocrit, %	46±4,0	46±4,0
MSP, mm^2	100±8.0	96±6.0
MSN, pg	29±2,0	29±2,0
MSNS, g/dl	290±16,0	320±28,0
Reticulocytes, %	4,2±0,3	6.2±0,2
Platelets,109/l	325±18,5	425±21,0
Leukocytes,109/l	3,4±1,1	4,5±1,0

Thus, the results of the conducted pharmacological studies, as well as the studies of other scientists [7] allow us to recommend polypeptides obtained by hydrolysis of the Bombyx mori cocoon for use as polymer carriers in the development of new promising dosage forms based on their polycomplexes with macro- and microelements.

The experimental part

Materials. The experiments used samples of a Bombyx mori cocoon after heat treatment grown in 2015 in Uzbekistan. Initially, caterpillars were extracted from the entire cocoon, then the cocoons were mechanically crushed into irregularly shaped pieces of 5-7 mm in size. NaOH, KOH, HCI and other chemical reagents of the H.H. brand were used in the work. All standards, buffers and chemical reagents used in the electrophoretic analysis method were purchased from Sigma-Aldrich (St. Louis, USA). Biochemical tests for morphological and biochemical studies were carried out using test kits manufactured by LLC "Pharmbiodiagnostics" (Tashkent).

Hydrolysis. The suspension in the amount of 5.0 g. of silkworm cocoon was washed with distilled water and placed in a conical flask with a volume of 250 ml, then filled with an alkali solution. The scales of the brand: KERN of the company AC/ACS (Germany) were used in the work. After mixing, the lid was closed and the flask was placed in a thermostat preheated to the required temperature (brand: MWB 20 (Germany). After passing the required hydrolysis time, the flask was removed from the thermostat and cooled to room temperature. The pH of the solution was determined before and after hydrolysis on a pH meter of the brand: EC-170 by pH-mV-Temp (Singapore). Before analyzing the hydrolysis product, it was neutralized by bringing the pH of the solution to the values of 6.8-7.2 by adding an aqueous solution of concentrated HCI.

Electrophoretic analysis. Electrophoresis of neutralized solutions (pH = 6,8-7,2) of polypeptides obtained by hydrolysis of the Bombyx mori cocoon in accordance with the described method with minor changes was carried out according to the method [6]. A 30 % aqueous solution of gel obtained by radical polymerization of acrylamide with N,N-bisacrylamide (0.034 % by weight of acrylamide) in water at a temperature of 333K was used as the solution, ammonium persulfate (0.25 by weight of acrylamide) was used as the initiator.

Pharmacological research. Pharmacological studies were carried out on samples of polypeptides obtained by hydrolysis of the Bombyx mori cocoon with NaOH solutions. The study of "acute" toxicity was carried out on white mice, of both sexes, weighing 18-21 g., 6 animals in each group. The test drug was injected inside with a special probe in doses of 1000 - 5000 mg/kg. After a single administration of the drug, observation was conducted hourly on the day of administration, 3 times a day for 2-3 days and once a day for the next 14 days of the experiment. The general behavior, coat color, mucosal condition, respiration, heartbeat, motor activity and death of mice were taken into account [8].

The study of the skin-irritating effect was carried out on 15 rats weighing 140±10g, which were applied 5 times subcutaneously to two right-cut areas of the skin of the back (2x2 sm) with an automatic pipette distilled water (control), and in the two left 0.5 and 5 % sample solution in a volume of 0.05 ml. The assessment of the local effect was carried out on the basis of the data of the examination carried out 4 hours after the introduction of the drug and the subsequent 7 days of the experiment [4]. The skin reaction was taken into account according to the scale of skin tests in points [5].

The authors thank the employees of the state enterprise "Center of Advanced Technology" (Tashkent) and the testing center "Pharmacological and Pharmacodynamic Research" of medicines at the IBOC of the Academy of Sciences of the Republic of Uzbekistan for their technical assistance in conducting, analyzing and pharmacological tests of the products of hydrolysis reactions of the cocoon Bombyx mori.

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