Uzbekistan Journal of Polymers
journal homepage: https://uzpolymerjournal.com/
ISSN (online): 2181-3256 (print) 2181-3246

POLYAMPHOLYTIC PROPERTIES OF PROTEIN FROM BOMBYX MORI SILKWORM PUPAE

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ARTICLE INFO

Received: 05 March 2025
Revised: 01 July 2025
Accepted: 08 July 2025

Keywords:
bombyx mori protein solution, isoelectric point, polyampholyte macromolecule, hydrodynamic studies

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ABSTRACT

The polyampholytic properties of *Bombyx mori* silk protein were studied using conductometry, viscometry, and potentiometry methods. During the titration of a 1% protein solution by the conductometry method, an increase in electrical conductivity (G_{sm}) was observed in a linear manner within the NaOH solution volume range of V = 4.4 – 5.4 ml, which was determined to correspond to the titration of up to 4.7% of the amino groups. When the viscosity of a 2% aqueous protein solution was examined using the viscometry method, an anomalous change characteristic of polyampholytic properties was observed upon dilution with water. However, when added with a 2% NaCl solution, this anomaly was eliminated, and the characteristic viscosity was determined to be [η] = 0.13 dl/g. Conformational changes in the protein chain were analyzed by potentiometric titration of 1% *Bombyx mori* alkaline hydrolysate (pH 11.9) with 38% acetic acid.

As the pH of the protein hydrolysate transitioned from alkaline to acidic through neutral conditions (pH 11.9–2.7), anomalous changes in the relative viscosity (η_{rel}) of the solution were observed. This was attributed to conformational changes in the protein, which resulted from the ionization of its basic (NH^+) and acidic (COO^-) groups, characteristic of polyampholytic properties. The analysis revealed that *Bombyx mori* protein comprises 18 amino acids, accounting for 28% of the dry weight (280.546 mg/g), composed of: 10 nonpolar amino acids (113.4 mg), 3 uncharged polar amino acids (82.6 mg), 3 basic amino acids (15.8 mg), 2 acidic amino acids (68.7 mg).

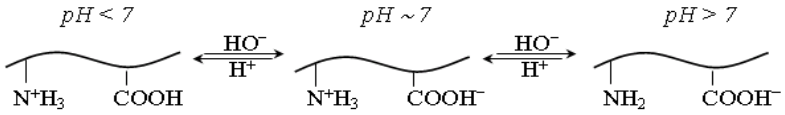
Introduction

Polyelectrolytes are characterized by their high density, with ionogenic groups present in each chain segment. In proteins, amino and carboxyl ionogenic groups appear after every 6 to 8 amino acids. This structure in polyelectrolyte chains generates electrostatic interactions in solutions, leading to highly flexible protein chains with significant deformation properties.

When both acidic and basic groups are present in a macromolecular chain, the solution exhibits polyacidic or polybasic characteristics depending on the pH level. Such polyelectrolytes are also referred to as polyampholytes [1]. In an acidic medium (pH < 7), the dissociation of acidic groups is significantly reduced, while the dissociation of basic groups transforms the macromolecule into a polycation, giving it a positive charge.

Conversely, in an alkaline medium (pH > 7), acidic groups primarily dissociate, resulting in a negatively charged macromolecule. In the intermediate range (pH ≈ 7), the polyampholyte macromolecule behaves like a bipolar (dipolar) ion.

These transformations can be expressed as follows [2]:



Proteins exhibit polyampholytic behavior due to the presence of both carboxyl (-COOH) and amino (-NH_2) groups. However, the presence of multiple ionogenic groups in proteins leads to variations in the manifestation of polyampholytic properties. Each acidic and basic group is characterized by a specific pK value, and even the same type of group can correspond to multiple pK values. The acidic properties of proteins tend to be more pronounced than their basic properties [3].

Protein chain flexibility is governed by the ionization state of its groups, pH, and the presence of low-molecular-weight electrolytes. High-molecular-weight electrolytes dissolve in polar solvents.

Protein macromolecules form stable lyophilic colloidal solutions in aqueous media. Similar to other proteins, *Bombyx mori* protein exhibits polyampholytic properties due to the ionization of its amino and carboxyl groups in solutions.

The polyampholytic nature of *Bombyx mori* protein solutions allows them to participate in reactions across different pH environments and interact with various molecules. Polyampholytic properties play a significant role in the biological and chemical characteristics of proteins [4].

Method

The quantitative characterization of amino groups in Bombyx mori protein was determined using the conductometric titration method. This study was conducted using a HANNA Instruments EC 215 conductivity meter, manufactured in Germany. Changes in electrical conductivity (Gsm) were observed concerning the volume (V) of the titrant—0.5 N NaOH, while sample solutions were prepared in the presence of 0.1 N HCl. The relationship between Gsm and V revealed characteristic titration points corresponding to specific amino groups within the protein chain. The viscometry method was employed to determine the relationship between protein concentration (C) and viscosity-related characteristics, based on Huggins' principles [5].

$$\eta_{rel} \approx [\eta] + k[\eta]^2 C \qquad (1)$$

Here, η_{rel} - represents the specific viscosity of the solution, while [η] denotes the intrinsic viscosity of the protein. The Huggins coefficient (k) is an indicator that reflects the presence of the polyelectrolytic effect across a wide range of concentrations (C).

The potentiometric titration method was used to determine conformational or structural changes in protein molecules. This process involved measuring the relative viscosity as a function of pH using a pH-150MI meter. Depending on the number of ionogenic groups, particularly active amino and carboxyl groups, various conformational states characteristic of polyelectrolytes were identified in the macromolecular chains.

The quantitative determination of amino acid composition in protein samples was carried out using an amino acid analyzer (HPLC Technologies 1200 with DAD detector) via high-performance liquid chromatography.

Prior to analysis, lyophilized samples (50 mg each) were hydrolyzed in 5.7 N HCl at 110°C under vacuum conditions for 24 hours. The resulting hydrolysates were evaporated using a rotary evaporator (DLAB RE 100-Pro) [6].

Results and discussion

Most protein molecules have a charged surface due to the presence of free COO^- and NH_3^+ groups. These charged ion chains interact with each other or with the dipole molecules of water,

leading to the formation of a hydration shell around protein molecules. However, water molecules in the hydration shell exhibit reduced mobility compared to bulk water, increasing their susceptibility to external perturbations, which can lead to protein precipitation.

During the precipitation of protein from a 1% alkaline protein hydrolysate, various physicochemical factors influence the properties of the protein sample, leading to changes in the macromolecular structure. To achieve effective protein precipitation from the hydrolysate obtained by deproteinizing *Bombyx mori* silkworm pupae, acetic acid was used to neutralize protein charges and break the hydration layer. The precipitated protein was dialyzed to remove low-molecular-weight compounds and then lyophilized at -50°C.

To determine the amino acid composition and quantity of the protein sample, high-performance liquid chromatography (HPLC) analysis was performed. The results are presented in Table 1. During the HPLC analysis, asparagine and glutamine were converted into aspartic and glutamic acids due to the influence of hydrochloric acid.

Table 1

№	Amino Acid Names	Concentration (mg/g)
1	Aspartic acid	40.448
2	Glutamic acid	28.264
3	Serine	8.913
4	Glycine	26.462
5	Cysteine	5.556
6	Threonine*	62.729
7	Arginine	2.000
8	Alanine	30.612
9	Proline	15.396
10	Tyrosine	10.954
11	Valine*	2.782
12	Methionine*	2.762
13	Histidine*	3.811
14	Isoleucine*	7.403
15	Leucine*	20.095
16	Tryptophan	0.000
17	Phenylalanine*	2.353
18	Lysine*	10.003
	Total	280.546

Amino acid composition of *Bombyx mori* protein. Essential amino acids are marked with an asterisk (*)

The analysis confirmed that the *Bombyx mori* protein sample contains all 18 amino acids, including 8 essential amino acids (marked with *). This indicates that the studied protein has a high nutritional value and quality.

Additionally, the total protein content in the sample was determined to be 28% (280.546 mg/g), distributed as follows:

- 10 non-polar amino acids: 113.4 mg
- 3 uncharged polar amino acids: 82.6 mg
- 3 basic amino acids: 15.8 mg
- 2 acidic amino acids: 68.7 mg

These amino acids exhibit polyelectrolyte (polyampholytic) properties, depending on the pH of the surrounding solution. The high acidity of the protein was analyzed using conductometric titration, which is based on the interaction of bipolar ions with sodium hydroxide solution, leading to specific reaction patterns.

$$H_3N + CH_2COO^- + Na^+ + OH^- \rightarrow H_2NCH_2COO^- + Na^+ + H_2O$$

As a result of the reaction, the electrical conductivity of the solution gradually increases due to the formation of the $H_2NCH_2COO^-$ ion (while the solution's resistance decreases).

After reaching the equivalence point, the excess sodium hydroxide (NaOH) causes a sharp increase in electrical conductivity (a drop in resistance) due to the increasing concentration of fast-moving OH^- ions. The change in electrical conductivity (Gsm) was monitored depending on the titrant volume (V). In this study: 0.5 N NaOH was used as the titrant. The titration was conducted in a 0.1 N HCl solution containing 1% *Bombyx mori* protein.

The Gsm–V dependency graphs show characteristic titration regions, corresponding to specific groups within the biopolymer chain. The provided graph (Figure 1) helps identify certain volume intervals of the titrant (V) that correlate with the titration of $-NH_3^+$ groups.

Based on the obtained data, the amount of amino groups (NH_2) per unit mass (m) of the protein sample was calculated using the following formula:

$$\omega_{(NH2)} \approx C_н m_{NH2} \Delta V_N / m \qquad (2)$$

Here: - C_n – normality of the titrant.
- mNH_2 – molecular mass of the amine.
- V_n – volume of the titrant consumed during the titration process.
- m – mass of the analyzed protein sample.

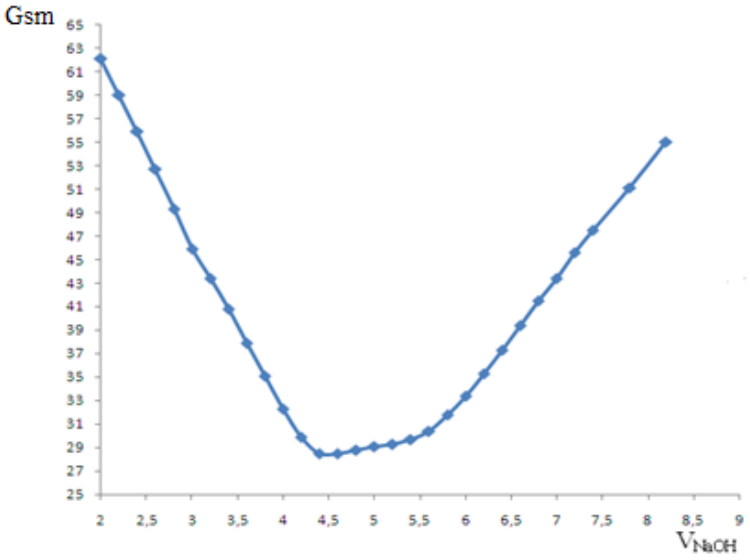


Figure 1. *Dependence of protein conductivity (G, mS/cm) on NaOH titrant volume (V, mL). The arrow indicates the equivalence point."*

Research results: Initially, with the first addition of the NaOH solution, conductivity significantly decreased, indicating the presence of excess HCl in the solution. In the titration volume range of $V_N = V_{NaOH} = 4.4 – 5.4$ mL, a linear increase in conductivity (Gsm) was observed, corresponding to the titration of amino groups. When $V_{NaOH} > 5.4$ mL, the presence of excess NaOH was confirmed, leading to a sharp increase in Wsm. Based on the experiments, the amount of NH_2^+ groups in the 1% *Bombyx mori* protein chain was calculated using formula (2) and found to be 4.7%.

The presence of hydrogen bonds and hydrophobic groups prevents the complete dissolution of the protein in water. To achieve full dissolution, the addition of a neutral salt (e.g., NaCl) is necessary, as it helps to shield the hydrophobic groups. In the absence of a neutral salt, phase

separation occurs over time, forming a high-molecular-weight fraction that remains undissolved and a low-molecular-weight fraction that dissolves. To evaluate the behavior of protein molecules in solution, hydrodynamic studies were conducted at room temperature using the "Ubbelohde" viscometry method.

Based on the obtained results, a graph (Figure 2) was constructed. When the viscosity of a 2% aqueous protein solution was studied by viscometry, an anomalous deviation (a) corresponding to polyampholytic properties was observed due to dilution with water, deviating from Huggins' law.

However, when a 2% NaCl solution (b) was added to the protein solution, this anomaly was eliminated and the intrinsic viscosity [η] was determined to be 0.13 dl/g. This confirmed that the charges and hydrophobic groups present in the protein chain were shielded, leading to a viscosity decrease following a linear trend according to Huggins' law.

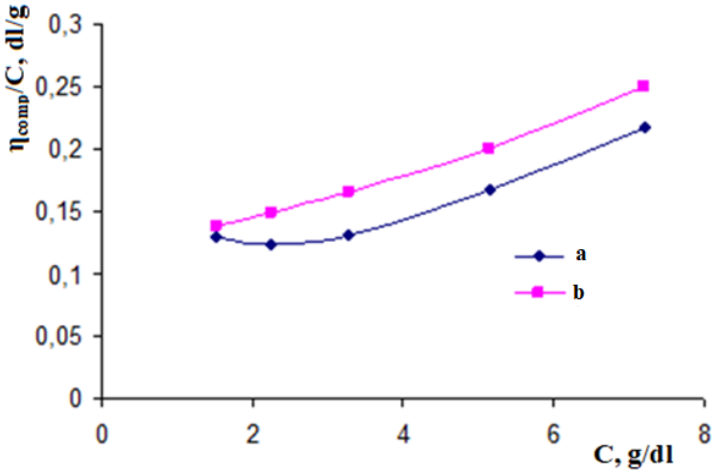


Figure 2. The dependence of the viscosity of the *Bombyx mori* protein solution (η_{comp}/C) on concentration (C) in (a) water and (b) with the addition of 2% NaCl.

Globular proteins, despite possessing a certain amount of layered *β*-structure in water, tend to lose their structure because their molecules contain more hydrophilic groups than hydrophobic ones. During the process of protein aggregation or globulization, structural degradation increases under the influence of various factors. However, this process is not affected by an increase in concentration and the resulting enhancement of intermolecular binding forces. In highly diluted solutions, the addition of protein leads to a decrease in viscosity, meaning that distilled water has relatively higher viscosity. As concentration increases, viscosity rises due to hydrophobic interactions and polar intermolecular forces.

In aqueous electrolyte solutions, proteins exhibit ampholytic properties. Although the pH of *Bombyx mori* protein is below 7, its amino acid composition (as shown in Table 1, HPLC analysis) determines its isoelectric point (IEP) at pH 4.8.-5.0 At this point, the ionized acidic groups (COO^-) and ionized basic groups (NH_3^+) are balanced. This equilibrium can be conventionally represented as follows.

$$OH^- + NH_3^+\text{-R-}COO^- + H^+$$

At the isoelectric point, despite the presence of some ionized groups in the macromolecules, the protein exhibits a neutral state. This occurs because the ionization of acidic and basic groups is balanced, leading to maximal internal attraction between them, which results in the compaction of the flexible protein chain.

Consequently, viscosity decreases as the chain adopts a static probabilistic globular shape, reaching maximum entropy. The conformational changes in the *Bombyx mori* protein chain were studied by titrating a 1% alkaline hydrolysate (pH 11.9) with 38% acetic acid.

Based on the obtained results, a graph was constructed showing the dependence of relative viscosity on the pH environment (Figure 3).

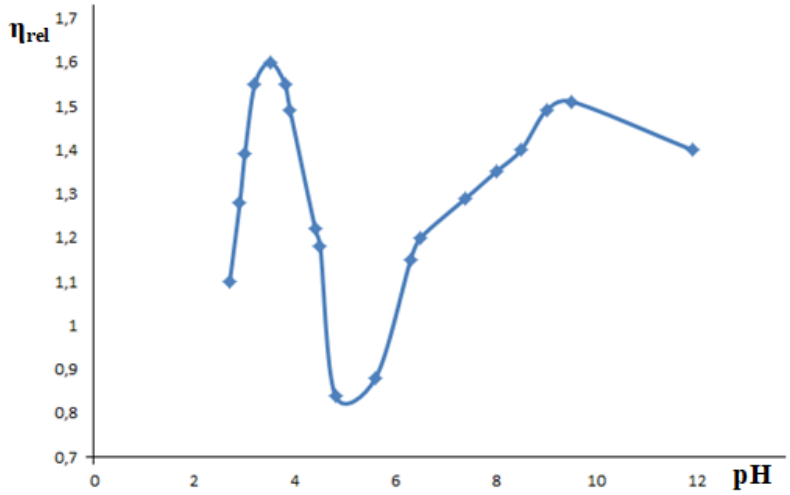


Figure 3. Graph showing the dependence of the relative viscosity of *Bombyx mori* protein on the pH environment

At the initial stage of titration, when the pH was -11.9, due to the significantly high amount of alkali (NaOH) in the solution, the protein formed a sodium salt, meaning that the excess Na^+ ion neutralized the ionized COO^- group: $HONH_3$-R-COONa.

Conformational density was observed in the protein chain, resulting in a decrease in relative viscosity (η_{rel} = 1.4).

When a certain amount of acid was introduced into the solution and the pH reached -9.5, the relative viscosity increased to η_{rel} = 1.51. At this stage, acetic acid combined with the Na^+ ion present in the solution to form sodium acetate, and as the amount of neutralized compound decreased, an increase in carboxyl group ions (COO^-) was observed.

The formation of an extra charge in the solution caused the polymer chain molecules to straighten, leading to a decrease in molecular density, partial expansion, and an increase in viscosity.

The isoelectric point (IEP) is not dependent on the concentration of polyampholytes. Parameters such as the swelling degree, solubility, osmotic pressure, and viscosity of the solution pass through a minimum at the IEP [7-10].

As protein hydrolysate titration continued and the pH of the medium reached -4.8, the graph showed that at the protein's isoelectric point, the relative viscosity reached its minimum value (η_{rel} = 0.84). This was because the amount of oppositely charged groups in the solution reached equilibrium and neutralized each other, leading to a more compact conformation of the flexible protein chain.

$$CH_3\,COO^- + NH_3^+\text{-}R\text{-}COO^- + H^+$$

When a shift occurs from the isoelectric point (IEP), for example, towards an acidic environment, the ion acquires a positive charge, and when shifted towards an alkaline environment, it acquires a negative charge.When the acidity of the solution increases and the pH reaches 3.5, the concentration of hydrogen ions rises, binding to the ionized carboxyl groups. As a result, the amount of ionized carboxyl groups (COO^-) decreases, while the number of ionized amine groups (NH_3^+)

increases. The relative viscosity reaches its maximum value at η_{rel}= 1.6. An increase in the like charges (amino groups NH_3^+) leads to a straightening of the chain conformation. This process can be conditionally expressed as follows.

$$CH_3\,COO^- + NH_3^+\text{-R-COOH} + H_2O$$

A sharp increase in acid concentration in the solution (pH 2.7) leads to an excess of CH_3COO^- ions, which combine with NH^+ ions to form acetate salt. As a result of the partial neutralization of the protein chain, the relative viscosity decreases to η_{rel} = 1.1.

$$CH_3\,COONH_3\text{-R-COOH}$$

If a polyampholyte solution does not contain additional external ions, the dissociation of its intrinsic ionogenic groups determines the pH level, which is referred to as the isoionic point (IIP).

The charge of the macromolecule at this point depends on the nature and ratio of acidic and basic groups in the chain. If IIP = 7 (i.e., pH = 7), it corresponds to the isoelectric point (IEP). This indicates that the solution is in an electrically neutral state:

$$C\langle z\rangle + [H^+] - [OH^-] = 0 \qquad (3)$$

Here, C represents the molar concentration of the polyampholyte, and <z> denotes the average net charge of the polyampholyte in the isoionic state. When pH = 7, the net charge <z> = 0.

The isoionic (IIP) and isoelectric (IEP) points are distinctly observed in polyampholytes, including proteins. Considering both IIP and IEP, conducting hydrodynamic studies allows for the precise determination of molecular weight and conformational characteristics.

Conclusion

During the conductometric titration of a 1% protein solution, an increase in electrical conductivity (Gsm) was observed in the range of V = 4.4 – 5.4 ml of NaOH solution, revealing the presence of 4.8% amino groups. The viscosity of a 2% aqueous protein solution was studied using viscometry, showing anomalous changes characteristic of polyampholytic properties when diluted with water. However, this anomaly was eliminated in a 2% NaCl solution, with a characteristic viscosity of [η] = 0.13 dl/g. The conformational changes of a 1% *Bombyx mori* protein alkaline hydrolysate (pH 11.9) were analyzed using potentiometric titration with 38% acetic acid. The study revealed anomalous changes in relative viscosity (η_{relat}) between pH 11.9 and 2.7. It was determined that the *Bombyx mori* protein chain consists of 18 amino acids with a total protein content of 28% (280.546 mg), including: 10 nonpolar amino acids (113.4 mg), 3 uncharged polar amino acids (82.6 mg), 3 basic amino acids (15.8 mg), 2 acidic amino acids (68.7 mg).

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