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Extraction of Hemoglobin from Eisenia Foetida Worms

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ABSTRACT

Today, two serious issues threaten human life, such as water pollution with hydrogen sulfide and the emerging corona disease. The special structure of hemoglobin of worms can remove hydrogen sulfide and carry a large amount of oxygen at the same time, and therefore, it is a suitable candidate for solving these two problems. Accordingly, the hemoglobin of Eisenia foetida worms was extracted by homogenization and several centrifuges and filters, and then the dimensions of hemoglobin were measured by dynamic light scattering technique, and the concentration and number of disulfide bonds in hemoglobin were investigated. The results showed that hemoglobin purity after the third stage of centrifugation and the second filtration was 56.2 and 91.3%, the measured diameter was 155.5 and 596.9 nm, the concentration of hemoglobin was 0.015 mol/liter, and the number of disulfide bonds was 120, respectively. According to the results of the examination, the extracted hemoglobin, having a large number of disulfide bonds, can react with hydrogen sulfide, and having very large dimensions, it is suitable for carrying a lot of oxygen.

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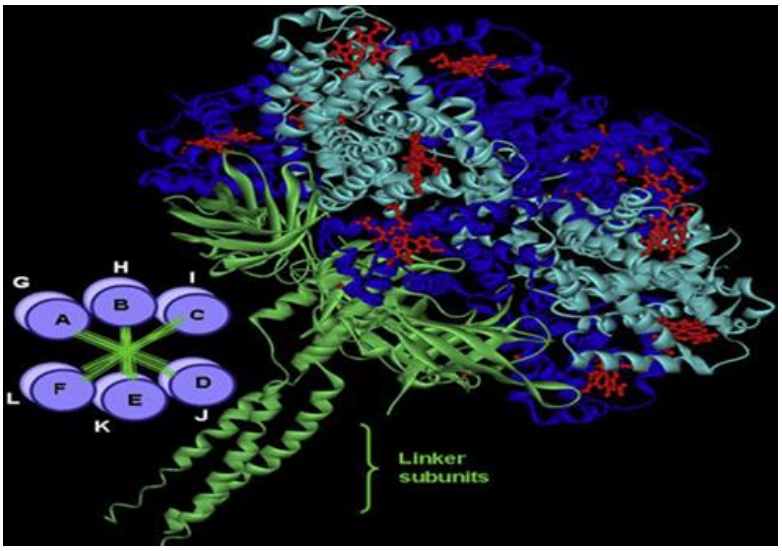
GRAPHICAL ABSTRACT



1. Introduction

The increase in human population on earth has led to an increase in household waste and sewage around the world. Sewage is often toxic and has a pungent odor due to the presence of H_2S. It has created problems for human life. On the other hand, newly emerging diseases, such as Corona, have caused the death of thousands of people by disrupting the supply of oxygen to the tissues. With the help of worm hemoglobin, we can overcome both problems to some extent so that one of the factors that we can use to remove H_2S from aqueous solutions and purify wastewater is the hemoglobin of live worms of Eisenia foetida. In addition, the extracted and purified hemoglobin of worms can be a good candidate for solving these problems related to oxygen supply in the human body. The researchers discovered that organisms in hydrogen sulfide-rich areas have special mechanisms to neutralize the toxic effects of hydrogen sulfide. These mechanisms include oxidizing enzymes or sulfide-binding amino acids [1]. One of the reasons for the absorption of hydrogen sulfide in these organisms has shown that the hemoglobin of the blood of these organisms, which is a protein structure, can bind to sulfide [2]. This protein is an extracellular hemoglobin and is found in three groups of worms, including annelids, Pogonophorans, and Vestimentiferans [3]. Morphological, genetic, and embryological studies have shown that these three groups are closely related [4]. Most of the information about the hemoglobin of these three groups was obtained from the study of the hemoglobin of the earthworm Lumbricus terrestris. The results of these studies are assigned to all groups [5]. The sulfide binding site in hemoglobin is different from the oxygen binding site [2]. To prevent the toxic effects of sulfide, these organisms take it with their hemoglobin and deliver it to the symbiotic bacteria in an organ called a trophosome.

1.1. Use of hemoglobin in biological processes

The study for the sequencing of extracellular hemoglobin, the structure of hemoglobin types, and the mechanism of hydrogen sulfide absorption by Riftia pachyphila hemoglobin have been perfectly done. Determining the amino acid sequence of extracellular hemoglobin of Tubifex tubifex worm [6] and complete amino

acid sequence of four globins in leech has been done[7]. The mechanism of sulfide binding to Riftia hemoglobin [8] and structural determinants for the formation of sulfohemoprotein complexes have been studied [9]. The study of the structure and function of globin based on its evolution [10], the architecture and structure of the hexagonal bilayer hemoglobin [11], the structural characteristics of hemoglobins [12], and the screening and preparation of genes related to sulfide metabolism in Riftia hemoglobin have been done [3]. Hemoglobin stabilization on nanoparticles, including zirconium dioxide nanoparticles, has been done to design a hydrogen peroxide biosensor [13]. In research, silica-coated iron oxide nanoparticles were bonded with 3-aminophenylbonic acid and connected to human hemoglobin for use in different techniques [14]. Seven fibrinolytic enzymes, eight glycosylated fibrinolytic proteases, and a component of lumbrokinase have been isolated from Eisenia fetida worms [15]. Spectroscopic studies of the reaction of iron oxide nanoparticles and human hemoglobin have been carried out in Iran. The results showed that the binding of maghemite nanoparticles to hemoglobin does not cause a change in the second structure and is a good guide for future designs [16]. Likewise, the biological absorption of hydrogen sulfide from water by live worms of Eisenia foetida has been successfully carried out [17].

1.2. Structural Characteristics of Hemoglobin of Worms

The hemoglobin of worms is large extracellular hemoglobin whose molecular weight is reported between 3000 and 4000 kDa [4,18,19]. The vermicompost worm Eisenia foetida and the earthworm Lumbricus terrestris are from the group of annelids [1, 2, 5]. The giant extracellular hemoglobin structure in these consists of two decamers. Each decamer contains one trimer and each trimer unit has one tetramer and each tetramer unit has two dimers and each dimer unit has four chains. In total, a double decamer has 144 globin chains (Figure 1) and 36 connecting peptides [5, 19, 20].

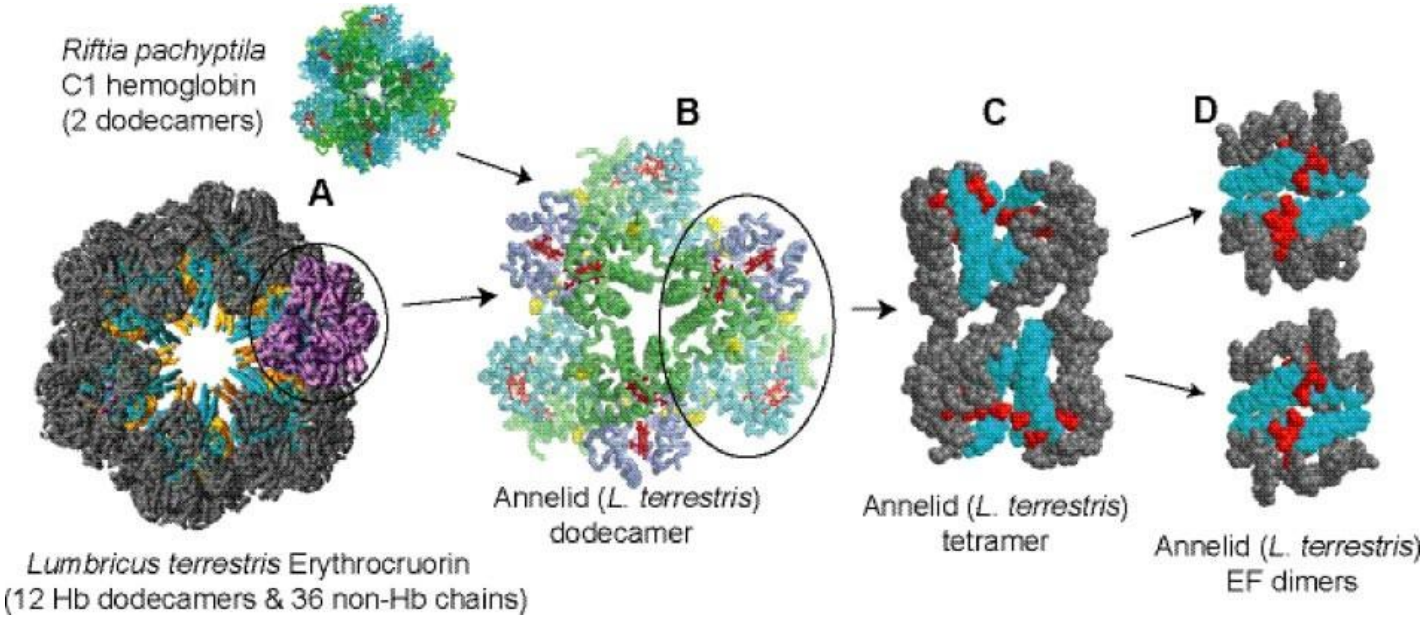


Fig1. Hierarchical subunit arrangement in giant extracellular hemoglobin of annelids [19]

Many researchers have reported hexagonal bilayer disk structures for this structure [21,22]. Electron microscopy studies have shown that hemoglobin is a multi-unit protein that has a hexagonal bilayer (Figure 2). Although its fourth building is not fully known, different models are considered for it including the bracelet model presented by the Vinogradov group and another hypothetical model presented by Rigo [11]. This bilayer hemoglobin model is called hexagonal. The most important issue is that although the large extracellular hemoglobin of Tobifax and Lambricus differed from Riftia hemoglobin in terms of molecular weight, they are both similar in terms of their disulfide bonds in the connecting chains and two free cysteines in globin. Free cysteines are cysteines that are not present in the structure of disulfide bonds and are exposed to carry out the reaction. The presence of disulfide bonds in globular proteins that are in extracellular conditions causes stability in these proteins [23].

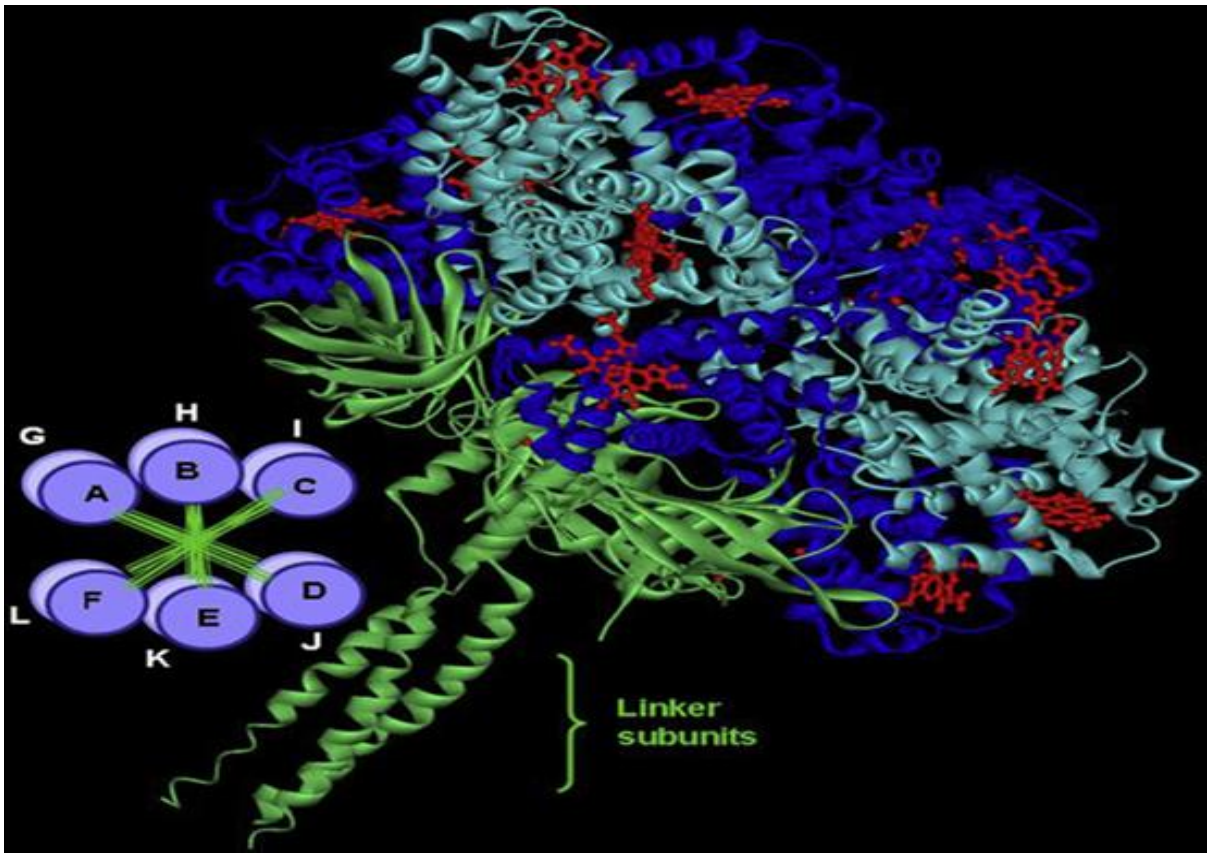


Fig 2. Hexagonal bilayer disc structures for obtaining extracellular hemoglobin [17, 22]

In recent research, this extracellular hemoglobin in annelids is called Erythrocruorin (Es) [19,24]. This hemoglobin in the body of these organisms can perform two functions at the same time. These two functions are oxygen transfer and hydrogen sulfide transfer [4,25]. Hemoglobin dimensions of Eisenia foetida vermicompost worm were measured by transmission electron microscope images and reported several times (Table 1) [17,26].

Table1. Hemoglobin dimensions of vermicompost of Eisenia foetida

Types of coloring	The distance between two parallel sides (nm)	Height (nm)
Uranyl Acetate	26	17.5
Phosphotungstate	26	16.5
Phosphotungstate	25	14

In addition, this hemoglobin is present in two different places in the body of the worm. These two locations include vascular blood and coelomic fluid. These two types of hemoglobin can bind to oxygen and hydrogen sulfide and transfer them to symbiotic bacteria [4,27]. In worms, sulfide is supplied by hemoglobin to symbiotic bacteria [4]. Oligobrachiama shikoi and Riftia pachyptila worms, which do not have a mouth, are fed by internal symbiotic bacteria that inhabit their trophosome. An intestinal study on vermicomposted Eisenia foetida showed that it contained 21 symbiotic bacteria [28]. Substances such as oxygen, hydrogen sulfide, carbon dioxide, and food are absorbed by the worm from the environment and delivered to the symbiotic bacteria through a vascular system in an organ called the trophosome. Chemolitoautotrophic bacteria coexist with them [27,29] which get their energy from sulfide oxidation [29].

1.3. The perspective of using worm hemoglobin to replace human blood

To replace human blood, extracellular hemoglobin known as Erythrocruorin is considered. These are evolved hemoglobin, so unlike human red blood cells that contain hemoglobin and have a membrane, they are membraneless and extracellular. For example, the common earthworm Erythrocruorin is a 3600 kDa giant complex consisting of 144 globin and 36 linker subunits. Each of the globin A, B, C, and $D_1/D_2/D_3$ has an intramolecular disulfide. While subunits A, B, and C have intermolecular disulfides that allow them to form a covalent ABC trimer. The bidecameric structure in globin is linked by the L_1, L_2, L_3, and L_4 linker units, which have their networks of disulfide bonds and complex helical domains, which allow them to assemble into a hexagonal bilayer that is 30 nm in diameter [30].

The results of the erythrocruorin study of earthworms and vermicompost have introduced them as two potential substitutes for human blood. Therefore, they need to study more in the future and it can be promising. They both resist oligomer dissociation at pH 7.4, while also exhibiting relatively low oxidation rates and high melting temperatures.

It is worth to note that these erythrocruorins have very different oxygen affinities, but the implications of this difference need to be determined in future animal studies. Finally, it

would be interesting to directly compare the erythrocruorins of the terrestrial organisms studied in this research with other erythrocruorins such as Arenicol marina [31].

1.4. The role of hemoglobin of worms in hydrogen sulfide

Invertebrates living in sediments are at high risk of exposure to hydrogen sulfide. Some of these invertebrates eliminate the risk caused by hydrogen sulfide by oxidizing the surrounding hydrogen sulfide with the help of nearby flowing water [31,32]. Of course, in areas rich in hydrogen sulfide, oxidation is not always effective. Among others, this issue is not effective in areas where the tide is low or in deep water or where the animal moves in the sediments or makes a new burrow. Therefore, in this situation, the animal should be resistant to hydrogen sulfide. Resistance to hydrogen sulfide, except in symbiotic species of hydrogen sulfide-oxidizing bacteria, may occur by one of the following mechanisms.

(i) Disposing of hydrogen sulfide, (ii) having cytochrome oxidase or pigments (heme) in the oxygen transport system that is insensitive to hydrogen sulfide, (iii) they depend on energy production with the anaerobic system, and (4) they eliminate the toxic effects of hydrogen sulfide that destroy. Toxic effects can be done by one of the following mechanisms.

(a) Oxidation of hydrogen sulfide and its transformation into sulfide with an enzymatic mechanism, (b) oxidation of hydrogen sulfide and its transformation into sulfide with a non-enzymatic mechanism through metal ions or metal protein complexes, and (c) inhibition of hydrogen sulfide by attaching it to metal ions and metal protein complexes in particular by binding to iron ions and glutathione tripeptide [33].

In some marine invertebrates, hemoglobin is involved in the transport of sulfide, for example, the large extracellular hemoglobin of the double-layered hexagon of the warm-water deep-sea tubeworm Riftia pachyptila simultaneously transports O_2 and H_2S in the blood from the gills to a special organ called the trophosome, which inside itself. It contains oxidizing symbiotic structures so that inside this organ, symbiotic bacteria use sulfide as the final acceptor of the electron transport chain in a metabolic pathway that converts carbon dioxide into sugar. There are three types pf hemoglobin in this animal, two types of hemoglobin V1, V2, vascular, and one coelomic hemoglobin C1 in this animal. Two types of vascular hemoglobin can bind to oxygen and sulfide at the same time, and this binding is reversible. The amount of hydrogen sulfide that can be transported by this hemoglobin is very high, depending on the environment; it ranges from 1 to 10 mmol/liter. Another example of hemoglobin with a high affinity for the thiol group of Lucina pectinate is observed. This tropical oyster has two types of Hb. The first is hemoglobin HbI, a monomer with 143 amino acids used to transport H_2S to autotrophic bacteria. The other two types of hemoglobin, HbII and Hb III, are dimer and tetramer, respectively, which are used for oxygen transport [22, 25, 34]. This article is one of the published articles related to the hemoglobin of worms. After the purification of hemoglobin, in this connection, the stabilization of the purified hemoglobin on sodium alginate and maghemite nanoparticles has been done, and then, the removal of hydrogen sulfide dissolved in water has been done using the stabilized hemoglobin on nanoparticles, which additional articles will be published in the future by the same authors.

2. Materials and methods

2.1. Extraction of worms' hemoglobin

Extraction of worms' hemoglobin was done according to Elmer's methods with some modifications. This method includes the following steps. The samples of worms used in

this research were purchased from one of the farms around Mashhad, and then they were transferred alive to the Research Institute of Applied Basic Sciences in a natural substrate containing cow manure. To conduct the research, the samples were separated from the natural breeding medium, and for 24 hours, they were placed in a container of water containing clean sieved sand to drain the residues of materials from the digestive system. The volume of clean sieved sand of one size was two-thirds of the volume of the storage container. After that, the worms were drained from the container, washed several times with water, and weighed with a laboratory scale (Figure 3).

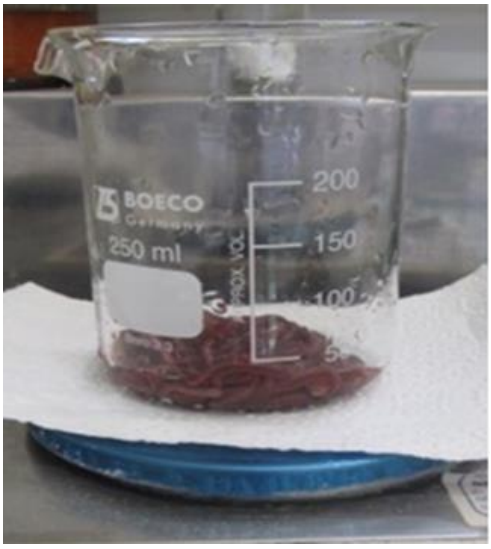


Fig3. The sample of worms in the cow dung and the state of weighing

In the next step, the worms were homogenized for half an hour until a uniform and homogeneous suspension of the worms was provided at the end. Next, the homogenous suspension prepared from the worms was centrifuged at 3000 rpm for 15 minutes, and then the supernatant solution was removed for the next steps and the remaining tissue parts were discarded (Figure 4). The supernatant solution was used to continue the extraction process of hemoglobin.

Fig4. Initial extraction of hemoglobin from tissue fragments

To prevent blood coagulation during the extraction process, a method similar to the one used by Yeh for the defibrination of sheep blood was used [35]. With this method, the enzymes and proteins associated with the blood were separated. The blood was poured into a beaker with glass pearls and stirred for 20 minutes with a rotary at 140 rpm, and then the blood is separated from the pearls (Figure 5).

Fig5. Preparation of pour blood from worms for hemoglobin extraction

In the next step, pour blood from the previous step was centrifuged at 6000 rpm for 30 minutes at 4 °C, and then they were centrifuged for two repetitions at 13000 rpm for 20 minutes at 4 °C. At each step, the supernatant solution was

removed and the sediment was discarded. On the other hand, after the end of the third stage of centrifugation, the hemoglobin suspension was separated from one of the Falcon tubes, respectively, from the top in the form of 10 tubes of one-milliliter vials. This was called the first extracted hemoglobin. In the continuation of the extraction process, the obtained blood sample containing hemoglobin was filtered using a 0.22-micrometer filter. Next, filtration was done using a 0.05-micrometer filter to complete the extraction process. This was called the second extracted hemoglobin (Figure 6).

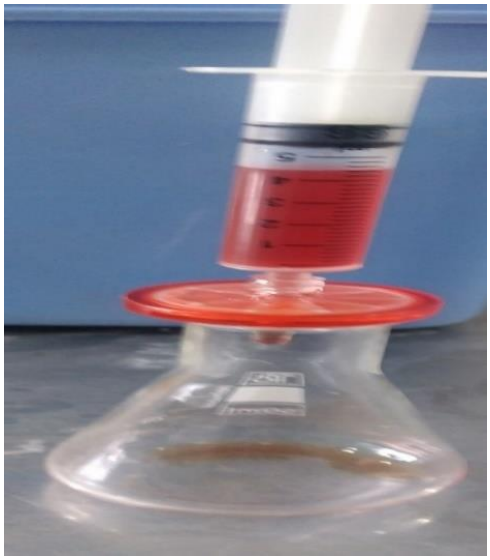


Fig6. Filtration of worm blood to purify hemoglobin

2.2. Structural studies of extracted hemoglobin

2.2.1. Determine the degree of purity of hemoglobin

To confirm and control the purifying of extracted hemoglobin from each step, we initially measured the absorption of the extracted product at a wavelength of 280 nm to determine whether the product contains protein, and then to confirm and control the presence of hemoglobin in the purified protein products obtained from each absorption step, the products were measured at a wavelength of 415 nm. By measuring the ratio of absorbance at 415 nm to absorbance at 280 nm, we calculated the purity of the obtained hemoglobin to some extent. In each stage of purification in the performed processes, the ratio of absorption in these two wavelengths was measured [36].

2.2.2. Hemoglobin and met hemoglobin concentration measurement

Hemoglobin concentration was measured according to the method of Dongott and Terwilliger. In this method, hemoglobin is diluted to 0.9% in physiological serum. However, the molar absorption coefficient of hemoglobin at the wavelength of 540 nm is equal to 14 $mmol^{-1}$. L cm^{-1}. With this method, they calculate the approximate concentration of hemoglobin. In this case, we have ignored the concentration of methemoglobin to accurately calculate the concentration of hemoglobin, and added 2.5 mmol/liter of potassium cyanide to the measured hemoglobin solution, and then we waited for 5 minutes at room temperature. During this period, CN^- anion binds to methemoglobin and causes the cyanohemoglobin production. However, the molar absorption coefficient at the wavelength of 540 nm is equal to 11 $mmol^{-1}$. L cm^{-1}. Meanwhile, we calculate the hemoglobin concentration from the difference in absorption in the second state and the first state [37].

2.2.3. Determining the number of disulfide bonds in hemoglobin

To determine the number of disulfide bonds, a comparative measurement method is used. Bovine serum albumin has 17 disulfide bonds. Accordnigly, under alkaline conditions; we measure the maximum absorption of bovine serum albumin at a wavelength of 335 nm, and then we measure the absorption of hemoglobin with the same concentration and conditions as bovine serum albumin. By comparing the ratio between them, the number of disulfide bands

was reported [8]. Thus, the amount of 0.500 g of bovine serum albumin was dissolved in 100 ml of deionized water, equivalent to the extracted hemoglobin concentration. Therefore, one volume of bovine serum albumin was diluted in two volumes of alkaline sodium carbonate buffer with a pH of 9.6. After that, the concentration of bovine serum albumin was measured at a wavelength of 335 nm. By knowing the number of disulfide bonds and the amount of absorption obtained from bovine serum albumin and the absorption of hemoglobin of worms, the number of disulfide bonds of hemoglobin of worms has been calculated, and then the isolated hemoglobin was subjected to dynamic light scattering analysis.

3. Results and Discussion

3.1. Extraction of hemoglobin of worms

The results of worm hemoglobin extraction showed that washing and emptying the digestive system of worms is very effective in accessing purified hemoglobin because the presence of environmental particles in the blood disrupts the process of hemoglobin purification. The use of a homogenizer is suitable for purification, although for the purification of small amounts of hemoglobin, homogenization with a mortar is sufficient. On the other hand, it is necessary to separate tissue parts from the blood by centrifugation at 3000 rpm. Using a glass pearl to prepare purified blood, coagulant proteins were removed from the homogenies of blood so that in the process continuation in the stages of filtration, extraction is not interrupted.

3.2. Determination of hemoglobin purity

The results of measuring hemoglobin absorption at a wavelength of 415 nm and 280 nm, which is protein absorption, showed that in the early stages of the process including after homogenization preliminary centrifugation, pearls treatment, two stages of high-speed centrifugation and the first stage of filtration, the percentage of hemoglobin absorption is somewhat similar to that of protein and it has been in the range of seven to 10% numerical value. On the other hand, when after the end of the third stage, the centrifuged hemoglobin suspension was separated from above in the form of one-milliliter vials; the purity level in the sixth treatment tube was equal to 60% (Figure 7). This was called the first purified hemoglobin. However, in the second stage of filtration, after the blood passes through the 0.05-micrometer filter, the absorption ratio of hemoglobin at a wavelength of 415 nm to protein at a wavelength of 280 nm or the purity coefficient has reached 91% (Figure 8).

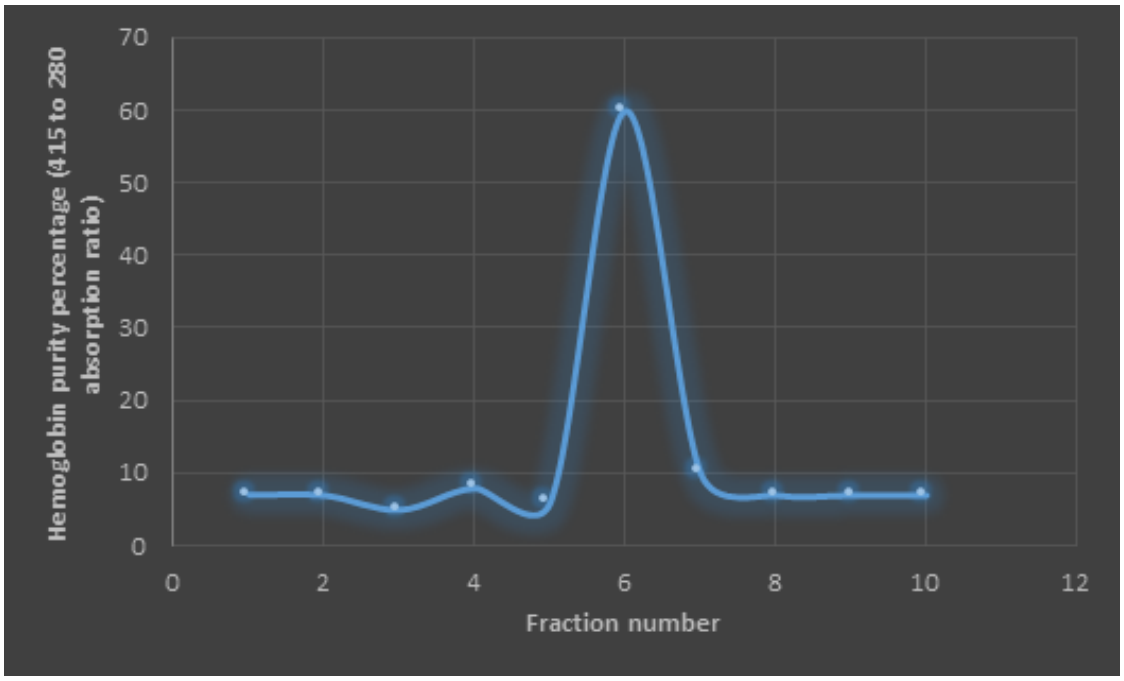


Fig7. Changes in hemoglobin absorption compared to protein absorption at 415 nm to 280 nm wavelength during the extraction process

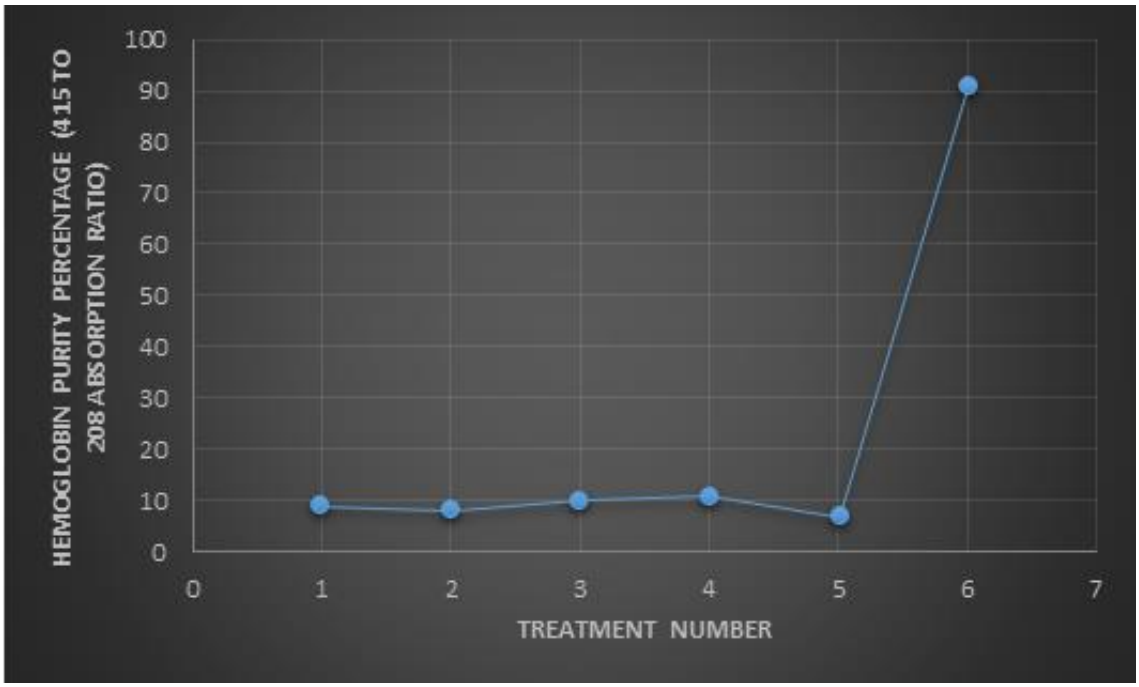


Fig8. Hemoglobin purity percentage by measuring the absorption ratio of hemoglobin at a wavelength of 415 nm to protein at a wavelength of 280 nm in the extraction stages ((1) Centrifuge 3000 rpm, (2) Pearls treatment , (3) Centrifuge 6000 rpm, (4) Centrifuge 13000 rpm, (5) 0.22 micrometer filter, and (6) 0.05 micrometer filter)

Changes in the percentage of purity in one-milliliter fractions after the third stage centrifugation were based on absorption from 415 nm to 280 nm. The results of measuring the dimensions of pure hemoglobin obtained by the dynamic light scattering (DLS) method showed that in tube number six at the end of the third stage of the centrifuge, (this was called the first extracted hemoglobin), the abundance purity was equal to 56.2% (Figure 9). This value is consistent with the results obtained from the absorption ratio at the wavelength of 415 to 280 with a purity of 60% (Figure 7). In addition, the results of the DLS at the end of the extraction stage, which used a 0.05-micrometer filter, (this was called the second purified hemoglobin), the purity percentage was equal to 91.3% (Figure 10), which was consistent with the results obtained (90%) from the ratio of absorption at the wavelength of 415 to 280. The results of the DLS showed that the diameter and width of hemoglobin were 155.5 and 35.56 nm, respectively (Table 2).On the other hand, in the hemoglobin extracted from the filtration stage, the hemoglobin diameter was 596.9 nm and its width was 328.2 nm (Table 5) so that the size of each hemoglobin unit is equivalent to four hemoglobin of the first stage and a tetrameric unit is formed and it is consistent with the report of the structure of this hemoglobin (Figure 10).

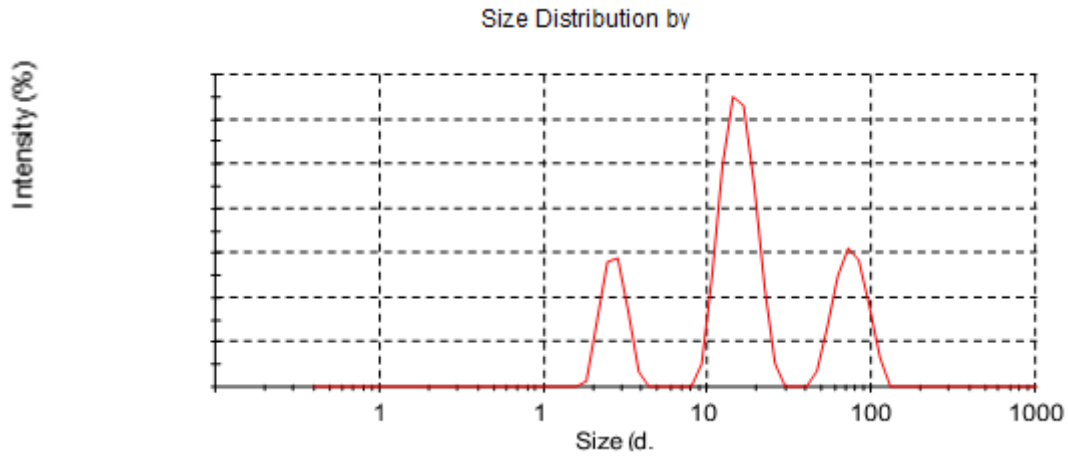


Fig 9. The results of dimensions and frequency of extracted hemoglobin (highest peak) of the first stage

Table2. Results of dynamic light scattering of extracted hemoglobin of the first step

Types of treatment	Peak number	Diameter in nm	Frequency percentage	Width
Hemoglobin	The highest	155.5	56.2	35.56
Associated Protein	Moderate	747.6	25.2	159.2
Associated Protein	The shortest	26.94	18.6	4.398

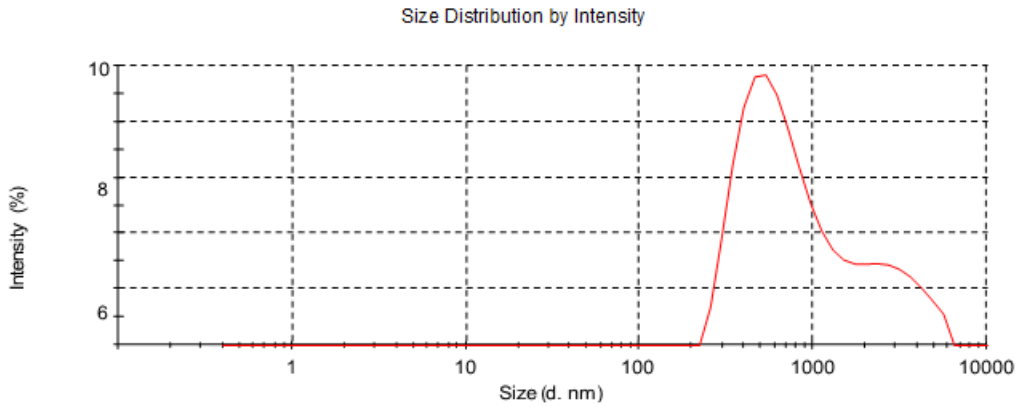


Fig10. The results of dimensions and frequency of extracted hemoglobin (highest peak) of the second stage

Table3. Results of dynamic light scattering of extracted hemoglobin of the second stage

Type of treatment	Peak number	Diameter in nm	Frequency percentage	Width
Hemoglobin	The highest	596.9	91.3	328.2
Associated Protein	Moderate	93.56	6.1	20.61
Associated Protein	The shortest	4844	2.5	706

3.3. Structural studies of extracted hemoglobin

3.3.1. Measuring the concentration of hemoglobin and methemoglobin

The results of the measurement of hemoglobin and methemoglobin concentration according to the Dongat and Terwilliger method show that hemoglobin concentration in the sample of the second extracted hemoglobin was 0.015 mol/liter. After the extracted hemoglobin suspension was exposed to potassium cyanide for 15 minutes, the concentration of hemoglobin suspension was equal to 0.016 mol/liter (Table 4). Without considering the absorption and with the calculated concentration, the met hemoglobin concentration is 0.001 mmol/liter.

Table4. Hemoglobin and methemoglobin concentration measurement results

Treatments	Absorbance at 540 nm	Concentration (mmol/liter)
A	0.214	0.015
B	0.171	0.016

Determining the number of disulfide bonds in hemoglobin

To determine the number of disulfide bonds, a reported comparative measurement method was used, according to the measurement of the absorption of bovine serum albumin and the absorption of hemoglobin extracted from worms, the approximate number of disulfide bonds in the hemoglobin of the worm was determined to be 120 (Table 5).

Table5. The results of determining the number of disulfide bonds in hemoglobin

Macromolecule	Absorbance at 540 nm	Molecular Weight (Kilo Daltons)	Number of disulfide bonds
Bovine serum albumin	0.068	66.4	17
Giant worm hemoglobin	0.479	3600	120

4. Conclusion

It is necessary to use glass pearls to prepare to pour blood. If the coagulant proteins and other proteins such as enzymes are not removed from the blood during the purification process, especially in the filtration stages, the associated proteins will cause the filter channels and pores to close and the extraction work will be interrupted, so to solve this problem, multiple filters must be used. Although the DLS results show that the use of glass pearls did not completely remove the accompanying proteins, compared to other results of worm hemoglobin extraction, these results were more favorable.

Chintan Savla *et al.* reported that they performed the extraction of earthworm Lt Ec hemoglobin using the TFF method. They emphasize that the quantitative assessment of the impurities along with hemoglobin, is the reason for their success in extraction. In addition, the reason for the failure in the extraction of hemoglobin in the protocols before them is due to the lack of quantitative assessment of the impurities accompanying hemoglobin [38-40]. Likewise, they consider the existence of a 40 kDa protein in the homogenate prepared from the Lt Ec as the reason for coagulation and as a result, the failure of hemoglobin extraction [40,41]. It is clear that Eisina fotida and LtEc worms of both genes are different, so it is normal to expect that the diameter of hemoglobin is not the same in them.

Different factors affect the results obtained from DLS, such as the presence of low molecular weight proteins in hemoglobin causing the difference in reported diameter. In addition, the presence of dust particles in the homogenate may cause a deviation of the reported diameter result in DLS as for LtEc hemoglobin[40-43].

30 and 28-diameter worms have been reported [1,40]. The diameter obtained by DLS conducted in the first extracted hemoglobin was 155.5 nm, which is different from the reported diameter of 28 for LtEc hemoglobin, which may be affected by the presence of proteins with high molecular weight. Furthermore, with higher purity, the DLS results will be the more accurate. The diameter obtained by DLS conducted in the second extracted hemoglobin was 596.9 nm so that in terms of dimensions, each hemoglobin unit is equivalent to four hemoglobin of the first extracted hemoglobin. Due to the delay in the DLS test and the fact that the hemoglobin sample has been kept in the refrigerator for four days until the DLS examination, a tetrameric unit has been formed and it is consistent with the report of the structure of this hemoglobin. It seems that in the *in vitro* environment, the functional units in the hemoglobin of Eisina fotida worm are put together with different formulas. It is not clear that they function as *in vivo*. According to the results of equalizing the concentration of hemoglobin and methemoglobin, it is likely that all hemoglobin and methemoglobin have been converted into cyanohemoglobin due to the

prolonged exposure time because the concentration of hemoglobin was below 10 mmol per liter. Hemoglobin and the exact concentration of hemoglobin have not been done. Regarding the number of disulfide bonds, the results seem to be acceptable, although there are few reports on the number of disulfide bonds.

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