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Original Article

A Study on the Mechanism of Aggregation of Therapeutic Reteplase Protein by Using the Monomer-Loss Model

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Abstract

Aggregation of pharmaceutical proteins reduces the efficiency and increases the cost of production. It can also lead to the reduced efficacy of drug or cause side effects on the patient's body. Investigating how to create them plays an important role to find agents that prevents the aggregation. This study was allocated for understanding the mechanism of formation of the reteplase protein using thermal stimulation. Aggregation was studied by ultraviolet spectrometry, and observation at 4, 25, 50 and 70°C, the concentration of protein monomer was measured by using a spectrum of 360 nm and 280 nm. At 4°C, there was no significant change in monomer concentration for a month. By increasing the temperature to 25˚C, aggregation process was slow, but at 70°C, the reaction was carried out at a rapid rate less than 2 hours. In order to investigate the mechanism of reteplase aggregation, some kinetics that was presented in of monomer-loss models were used. Experimental data was fitted in three "pre balance core", "self-catalytic" and "slow start" models using MATLAB. The best fit was obtained using optimization methods. Best fit for self-catalytic model is (R^2> 0.98). For other two models (R2 <0.9) occurred. The best fit for the pre balance core and a slow start model was occurred in n = 2. These results could indicate that the core is formed by connecting two reteplase monomers together. The reaction rate constants were calculated too. The results showed that increasing the temperature increases the reaction rate constant. With increasing temperature from 25 ˚C up to 70˚C, both of K1 and K2 increased from $4.7\pm0.1*10^{-11}K_1(min^{-1})$ to $1.6\pm0.2*10^{-7}K_1(min^{-1})$ and from $1.04\pm0.2*10^{-5}$ $K_2(M^{-1}$ $min^{-1})$ to $1.5\pm 0.3*10^{-4}$ $K_2(M^{-1}min^{-1})$, respectively for autocatalytic model. Limitation step of the reaction is the nucleation. $K_1<K_2$ demonstrates this fact. In addition, an existence of disulfide bonds in aggregate protein was guessed. These bonds are strong covalent that cannot be easily broken.

Keywords: Reteplase, Aggregation, Mechanism, Monomer-Loss Model, Thermal Stimulation, Nucleation

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Introduction

Vascular diseases of the heart and brain are the most threatening human diseases. More than twelve million cases worldwide lose their lives each year. Attention to the fact that blood coagulation occurs in many disorders, hepatic, renal and metabolic endocrinology, as well as common diseases, surgery, gynecology, stroke, acute myocardinal infarction and disseminated intravascular coagulation is important. Therefore, mechanisms for making effective drugs clot breaking and breaking seems mandatory [1]. Clot buster factors are drugs that have been developed quickly to remove clots and reduce damage to surrounding tissue damaged vessels [2]. These factors are biological drug that are produced by using recombinant technology. Recombinant expression increasingly has become important method for producing large amounts of protein for therapeutic and biotechnology applications. The recombinant protein product cause a huge change in the type and the variety of medicinal products consumed, even today, the recombinant pharmaceutical products with high molecular weight are more than small chemical molecules [3]. Advantages of the therapeutic proteins such as their economical perspective make them more attractive than the common drugs [4, 5]. Reteplase is the first fibrinolytic of third generation and the modified type of tissue plasminogen activator in non-glycosylated form in body [6]. It has 357 amino acid of 527 amino acid of main protein. This protein is produced by recombinant DNA, because of loss of carbohydrate and non-glycosylated chains, they can be expressed in bacterial cells such as *E.coli* [7]. This drug was produced in 1996 by FDA. Its' half life is more than other clotting factors and is about 13-16 min (Drug Bank). Often, recombinant proteins production becomes corrupted with its aggregation in host. The aggregate formation (accumulation), is a phenomenon that occurs particularly for unstable protein especially for pharmacy non-glycosylated proteins [8, 9].

Pharmaceutical protein accumulation at different stages of processing and the development of processes, including cell culture/fermentation, purification, formulation, transport, storage makes problem. Existence of the aggregate in final product causes potential adverse

physiological consequences. The aggregates can increase the changes and toxic and also stimulates the body's immune system. So, efforts and use of the resources for understanding and determining the type and the amount of aggregates for the development of biological agents with the aim of minimize the amount of the final product is very important. It is noteworthy that there is no single method for analyzing various forms of aggregates and often the choice of a method are done according to the size and characteristics of aggregates [10]. Explain the molecular mechanisms of the formation of aggregates are one of the central focuses of research to improve the therapeutic properties of proteins [11]. Also, understanding these mechanisms is a purpose that has a vital importance to reduce or prevent the formation of these aggregates in reteplase protein.

In order to understand the aggregation mechanism, the evaluation of the existing models is essential. During the last years some studies were done that different models are presented to express the kinetic form of the protein. In 2009, Bernacki and Murphy group classified some models for aggregation mechanism based on Monomer-loss. These models that are used for the description of aggregation kinetic are applicable in most of proteins and with simplifying assumption used for them, have simpler equation for other models. Heat increases the rate of aggregate formation. So it can be used as a stimulating factor for aggregation to study the mechanism of aggregation in reteplase protein solution.

Materials and Methods

Materials

Protein solution of Tosez produced in ZistDaru-Danesh Company was used. This solution contains reteplase protein solution in urea with 0.35 M concentration, 1molar urea pH=8, and 30 m $MKHPO_4$. Buffer solution (control) used has protein solution characteristics, but has not protein.

The solution of 10% (w/w) urea and dodesyle sulfate was used to identify and analyze the bonds between protein chains to produce aggregates.

Stimulating protein to form aggregate

To study the kinetics of aggregate formation, using heat as a factor in accelerating the formation of protein monomers is necessary.

Heating is a standard method for the stimulation of protein aggregation. For this purpose, samples of reteplase protein solution were kept in 4, 25, 50, 70ºC for several days. 4ºC was in refrigerator, 25ºC in autoclave, and 50ºC, 70ºC in oven. 50ºC, 70ºC were chosen in order to melting point of reteplase. Melting point of reteplase with respect to Drug Bank is 60.

Therefore, two temperatures close to this point, one above the melting point and the other below the melting point was chosen. Ambient temperature to evaluate the shelf life of aggregate in ambient and 4ºC for storing it in refrigerator was chosen.

Aggregation identification method

According to research conducted in the context of determining the mechanism of aggregate formation in proteins, several onstrumental methods are used for this purpose. Absorbance UV spectroscopy is classified as one of the absorbance methods. This method is one of the direct methods to obtain the kinetics of aggregation in reteplase protein. Based on this subject and experiments that was done to determine the method, finally, the UV spectroscopy was chosen as a suitable method. This method is one of the direct methods to obtain the kinetics of aggregate formation in the reteplase protein solution. Because of the high sensitivity, UV spectrometry is suitable method to investigate the accidents that occur during the process of protein. The strong absorption above 250 nm, especially in 280 nm that is related to the amino acids tyrosine and tryptophan, is important in measurement of protein. Cysteine amino acid is also has a known absorbance at 280 nm [12].

UV spectroscopy

If the light in the range of UV–visible of electromagnetic spectrum is passed through the solution containing the sample part of light energy may be absorbed and spent on some electron transitions. This act is done in absorption spectrophotometer or spectrometer.Increase in the optical density at 360 nm wavelengths and decrease in (OD280 nm)/(OD260nm) ratio indicate the aggregate formation in the protein solution [13]. For this purpose, the spectrophotometer was used in the laboratory.The device was calibrated by control solution, the buffer that has not protein. Then, after each step of heating, the sample was poured in a specific cell and the absorbance in selected wavelengths was studied. To measure the concentration of monomer protein in the solution, the absorbance at a wavelength of 280 nm was used.

Method of perform experiments

Study of the kinetics of the formation of reteplase protein based on temperature changes were performed at four different temperatures. The samples were placed at the desired temperature. As mentioned to read the absorbance of the sample spectrophotometer was used. Since the formation process was slow at 4°C; the absorption of samples was measured at 360 nm once a day. For a temperature of 25°C, the absorbance at 360 nm was read twice a day. But at high temperatures due to the increased speed of aggregation in the solution, measuring the absorbance changes at intervals was very limited. The absorbance for each time was read by a spectrophotometer and then continued in the same way until the absorbance was fixed. With each sample, another sample for reading the absorption wavelength of 280 nm was also under the temperature and during the experiments its' change was determined once or more. To read the absorbance at a wavelength of 280 nm, the samples were centrifuged, and then placed in a spectrophotometer. The absorbance at a wavelength of 280 nm was used to read a monomer protein concentration of the sample. To calculate the monomer concentration in the absorbance wavelength of 280 nm this equation was used. To confirm the results of measuring the absorbance at a wavelength of 360 nm, as measuring the

absorbance at a wavelength 280 nm, absorbance in nm 260 is measured using the ratio (OD280 nm) / (OD260nm).

$$\frac{Extinction\ coefficient}{\text{absorption wavelength of 280 nm}} = Monomer\ concentration\ of\ protein$$

Examining the type of forming the bonds of aggregate

Aggregate formed by the bonds, are created between the amino acids in the protein chains. These bonds can be covalent or non-covalent. If the bonds are in disulfide type, aggregation is covalent, and if bonds are hydrogenised or its interactions are hydrophobic, aggregation is non-covalent. To identify the type of chemical bonds, firstly, in several steps urea concentration that resulted in the loss of many non-covalent bonds, was increased in the aggregate solution. Absorbance of the solution was read at wavelengths of 360, 280 and 260 nm. In next step the 10% w/w of SDS solution that destroys stronger non-covalent bond as hydrogen bonds was added and the absorbance was read at a mentioned wavelength again.

Pre-equilibrated nucleus (PN) model

In this model, monomers (M) are in rapid equilibrium with a nucleus (N) containing n monomers. Then free monomers bind to the nucleus N,into was A aggregation irreversible reaction. Then the others monomers are connected to the mass and the reaction continues. Reaction paths as follows [14].

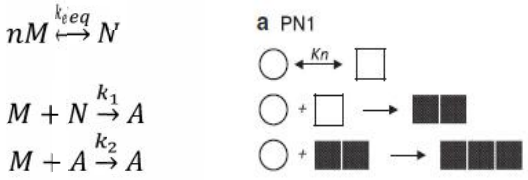


Figure 1. Schematic pre-equilibrated nucleus (PN) model

$k_{eq} = [N]/[M]^n$ is an equilibrium constant of first reaction. In this model, it is assumed that the growth rate (K_2) is independent of the size of aggregate. So if $k_1 = k_{eq}\, k_1'$ then:

$$\begin{cases} \frac{d[M]}{d[t]} = -k_1[M]^{n+1} - k_2[A][M] \\ \frac{d[A]}{d[t]} = k_1[M]^{n+1} \end{cases} \quad (1)$$

Autocatalytic (AC) model

In this model, the monomer of "M", in irreversible reaction, become irregular, nuclear is produced and in next step the aggregate is formed. Produced aggregate facilitates the production of other aggregates of other M monomer. In this model it is assumed that the reaction is carried out in two stages, which includes a slow continuous nucleation step and subsequent growth is rapid catalytic surface. If the reaction rate constant of first reaction is K_1 and the rate constant of second reaction is K_2 then [14, 15]:

Figure 2. Schematic autocatalytic (AC) model

Equation of above reactions is in the form of (2) relation.

$$\begin{cases} \frac{d[M]}{dt} = -k_1[M] - k_2[M][A] \\ \frac{d[A]}{dt} = k_1[M] + k_2[M][A] \end{cases} \quad (2)$$

If we assume that at t=0 $[M] = [M_0]_{@t=0}$ then:

$$[M] = \left\{{}^{k_1}/_{k_2} + [M_0]\right\} \Big/ \left\{1 + {}^{k_1}/_{k_{2[M_0]}}\, exp(k_1 + k_2[M_0])t\right\}$$

(3)

$$[A] = [M_0] - \left\{{}^{k_1}/_{k_2} + [M_0]\right\} \Big/ \left\{1 + {}^{k_1}/_{k_{2[M_0]}}\, exp(k_1 + k_2[M_0])t\right\} \quad (4)$$

Slow Initiation (SI) model

Against the pre equilibrium ***nucleus*** model, in slow initiation model, it is assumed that monomers irreversibly become aggregate and in next step monomers are added to the aggregate and the mass growth [14, 16].

Figure 3. Schematic slow initiation (SI) model

Pre-equilibrium core model was used for assumed same equilibrium equations.

$$\begin{cases} \frac{d[M]}{d[t]} = -nk_1[M]^n - k_2[A][M] \\ \frac{d[A]}{d[t]} = k_1[M]^n \end{cases} \quad (5)$$

Implementation of test data on the models

Mentioned models were used to measure and analyze data. For this purpose, the equations were programmed in MATLAB, and by fitting each model and solving it by numerical method for mentioned models, on experimental data, aggregation rate constants were calculated for each model. Also by optimizing the programming equations, it was studied that in which model the mechanism of aggregation for the reteplase protein solution is better.

Results and Discussion

The overall trend of the samples in the range of 360 nm absorbance, that is associated with read absorbance, and the reduction of ratio (OD280 nm) / (OD260nm) as it said in literatures, indicates the aggregate formation of a protein in solution. Also changes in the absorbance spectrum of UV for protein solutions express creation of the aggregate with irregular structure in protein solution. From data fit in monomer loss model for study aggregation mechanism in reteplase protein solution and the effect of temperature in it was used.

Results for 4ºC

For 4ºC, with the results of the experiments, as shown in figure 4, it can be observed over time in a month that the reduction in protein monomer concentration is minimal, so it seems, we can be able to keep not formulated reteplase protein in refrigerator temperature for a month.

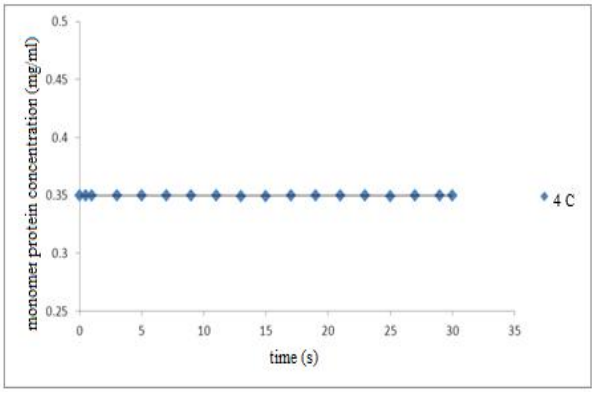


Figure 4. Change in reteplase monomeric protein in 4ºC. (The temperature of the refrigerator, in a month, there was no significant change in the reteplase monomer protein concentration)

The results of fitting the data in autocatalytic model

In examining the data fit for autocatalytic model, according to the figure 5, we can say that as the temperature increases, the formation of aggregated reteplase protein and the increase of its velocity are happened. According to the results, we can say that with increasing temperature the protein monomers lose their stability and their structure is changed. With these changes, incorrect folding and the creation of favorable conditions for them occur.

As can be seen in Table 1, At 70 ºC better fitting than the 25, 50ºC was obtained R^2> 0.98. Also R^2 for 50ºC was more than 25ºC. One can infer that as the temperature increase, the aggregation mechanism will have a better fitting with autocatalytic model. As mentioned in above, the autocatalytic model is formed from nucleation step and fast surface autocatalytic growth. It may be concluded that, because the temperature increases, nucleation is happen in less time, allowing the creation of the stage and the interaction of the two phases are reduced.

Table 1. Regression of fitting data in autocatalytic model for 25, 50, 70ºC.

Temperature (ºC)		
25	50	70
R^2< 0.77	R^2< 0.81	R^2< 0.792

This event causes in the nucleation stage, the increase in concentration doesn't seen and the autocatalytic growth phase occurs more rapidly. This trend with increasing temperature causes the better fit of the reteplase protein aggregation mechanism with autocatalytic model.

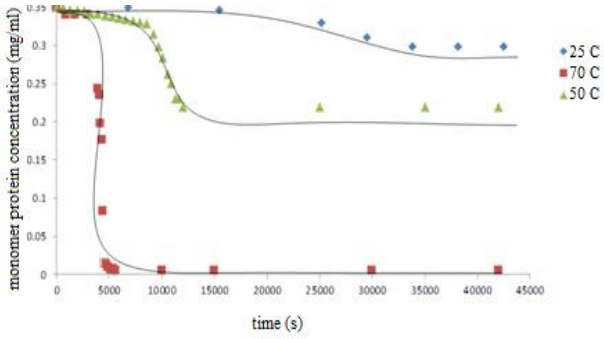


Figure 5. Lines of the best fitting, the effect of temperature on the aggregate formation rate of reteplase for model was obtained in 25, 50 and 70ºC.

According to figure 6a, b, and c, fitting the experimental data in pre equilibrium core model was happen in with less R2 than autocatalytic model. This model has the best fitting in n=2. Also, between models, the best fitting was for 50ºC with R^2, 0.989 in n=2 (Fig. 6b), (R^2 are given in Table 2). So in this model, unlike the autocatalytic model, data fit doesn't increase with increasing temperature. This conclusion is justified according to difference between these two models and assumptions that are intended for pre balanced model. With studying the mechanism of this model, this result can be interpreted. Since in the mechanism of pre-equilibrium core model, core and monomer are in equilibrium and K_1 is calculated $k_1 = k_{eq} k_1'$. In fact, at one stage of the mechanism, the protein solution contains monomers, core and protein aggregate. As figure 6b shows, because the phase nucleation occurs at longer, it is possible to create a small amount aggregate in the solution.

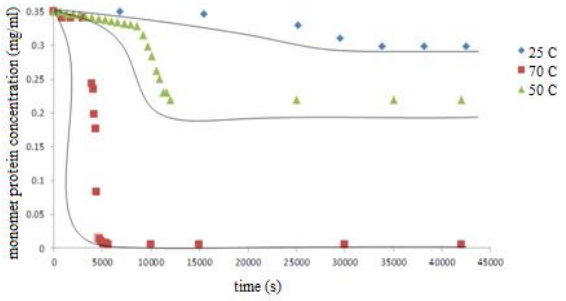
In the growth stage, because the reaction is slow and not complete, some monomer and protein core there are in the nucleus.

Table 2. Regression of fitting data in slow initiation model for 25, 50, 70ºC related to Fig. 6.

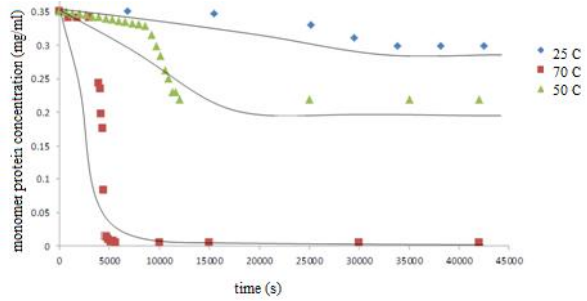
Temperature (ºC)	R^2 Fig. 6a	Fig. 6b	Fig. 6c
25	R^2 <0.841	R^2 < 0.860	R^2 < 0.820
50	R^2 <0.880	R^2 < 0.898	R^2 < 0.868
70	R^2 <0.864	R^2 < 0.881	R^2 < 0.842

While at 70 ºC, as it said for autocatalytic model, such a situation does not happen. In 25ºC also due to low concentration of protein monomer and length of time, was not a good fit such as for 50ºC. The better fitting in n=2, shows that the nucleus consist of two monomer protein.

a)



b)



c)

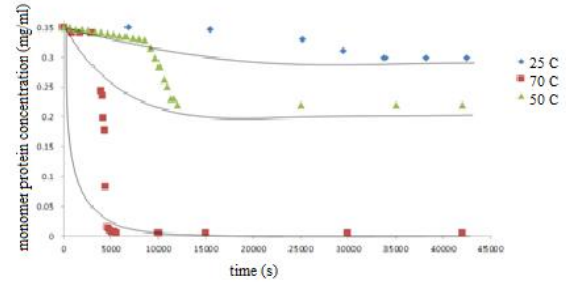


Figure 6. Lines for best fitting of effect of temperature on reteplase aggregation rate for pre equilibrium core model in 25, 50, 70 ºC; a: n=1, b: n=2, and c: n=3.

Fitting data for slow initiation model

In this step, nucleation was not happen. As shown in figure 7, the highest fitting is for 50ºC and n=2 with R^2< 0.8. Comparing the regression results in Table 3 also evident. Matching data in this model compared to the pre equilibrium core and autocatalytic model occurred with less regression. Due to the low compliance data for this model and that in this model the nucleation stage is not intended to aggregate, the existence of nucleation stage in the aggregation reaction enhances. As can be seen in figure the distance between the lines fitted to the data of the first stage is high but by going into the second phase of diagrams, especially at 50°C it is pulled to data. These results show an increase in the amount mass in the solution and going to the second stage of the reaction will increase the consistency of data in the model. Then the growth phase reaction occurs as expressed in the models.The curves obtained by fitting the data to the model showes that the change is ascending at the first and is omitted in nucleation (Fig. 7). The better result is happened in 50°C because of the existence of some aggregates in the nucleation step. Lower compliance at 25°C is due to the low reaction and changes in solution. The best fitting is happened in n=2. Such as result of pre equilibrium core model, that shows the initiation of aggregation by joining two reteplase monomer proteins together.

Obtained rate constants and their results

The models are fitted with coded models, for each model in the temperatures of 25, 50 and 70°C, the rate constant K_1 and K_2 were calculated, respectively.Rate constants with increasing temperature increased from 25ºC to 70ºC. This result indicates that the formation of the reaction rate increases with increasing temperature.

As Table 4, it can be seen that the rate of constant difference between 50 and 70ºC temperature is low. While there is a difference between the fixed rate at 25, 50ºC according to 50 and 70ºC, the temperatures near the melting point of reteplase, the amount of heat energy is spent on changing the phase of protein, and little of it will be spent on increasing the reaction rate. In this way, when passing the solution the temperature from 60 to 70ºC, the amount of energy spent on accelerating the aggregate formation reaction is reduced and the adherence of the rate constant of the reduction will be less. This happen causes the constant rate in 50 and 70ºC be close together. By far from melting point some more changes will happen. Distances between rate constants in 50 and 25ºC confirm this point. Comparing the rate constant in first stage of reaction (K_1) with second (K_2), was done after assimilating the units.

Results show K_1 is lower than K_2 for 3 models. Smaller rate constant represents the first stage of this process is slower than the second stage. Due to the limiting step of the reaction, the step that reaction takes place in lower rate and rate constant is lower, After the reaction, nucleation stage, due to a lower rate and the lower rate constant, the limiting step of the reaction.

Investigating bonds that causing aggregates

It was observed that by adding urea and sodium dodecylsulfate 10% w/w no change in the optical

absorbance was happened. This two materials are Annihilator of the non covalant bonds in solution.

Table 3. Regression of fitting data in slow initiation model for 25, 50, 70ºC related to Fig. 7.

Temperature (ºC)	R^2 Fig. 7a	Fig. 7b	Fig. 7c
25	R^2 <0.739	R^2 < 0.770	R^2 < 0.728
50	R^2 <0.794	R^2 < 0.810	R^2 < 0.780
70	R^2 <0.769	R^2 < 0.792	R^2 < 0.762

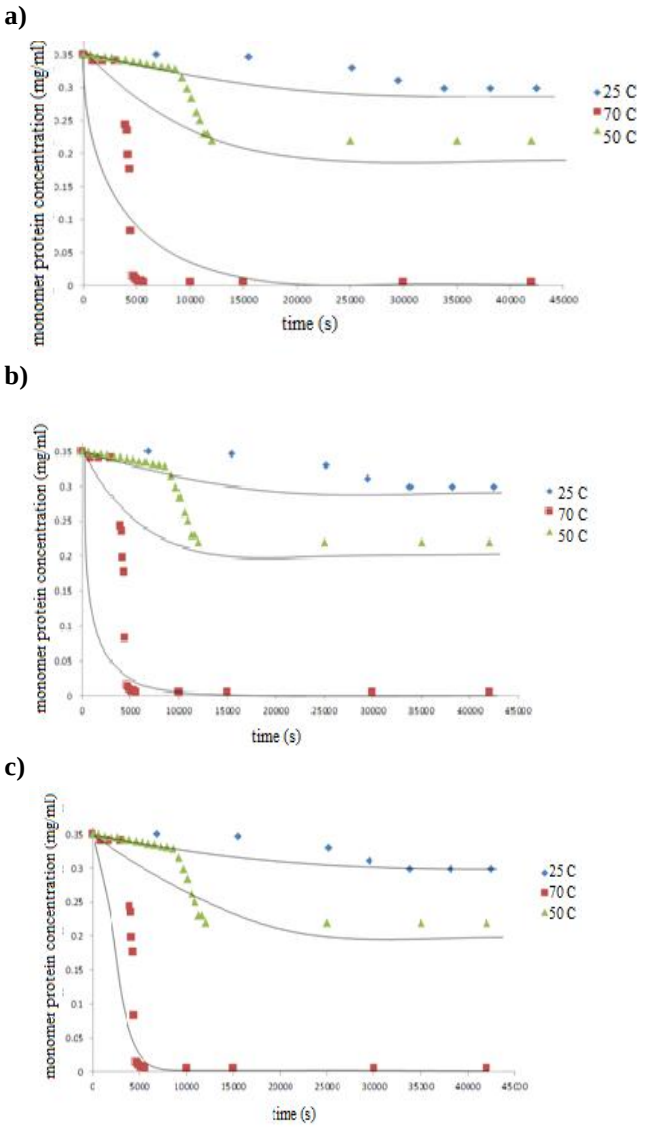


Figure 7. Lines of best fit, the effect of temperature on the rate of formation of reteplase aggregate for slow initiation in the three-temperature models of 25, 50, 70ºC; a: n=1, b: n=2, and c: n=3.

So no change in absorbance read, indicatesthat thenon covalant bonds are notpresent in the solutionand bonds fasteners monomers of Reteplase that are Cause aggregation,of type covalant [17-20].

Table 4. Nucleation and growth rate constants (K_1, K_2) for the aggregation of reteplase at different temperatures for pre equilibrium core, autocatalytic and slow initiation models

Temperature (ºC)	K_1 autocatalytic ($M^{-1} min^{-1}$)	Slow initiation ($M^{-1} min^{-1}$)	Pre-equilibrium core ($M^{-1} min^{-1}$)
25	$1.04\pm0.2*10^{-5}$	$6.3\pm0.2*10^{-5}$	$1.1\pm0.1*10^{-5}$
50	$1.3\pm0.2*10^{-3}$	$1.5\pm0.1*10^{-4}$	$1.0\pm0.3*10^{-3}$
70	$1.5\pm0.3*10^{-3}$	$1.1\pm0.1*10^{-3}$	$4.6\pm0.1*10^{-3}$

Temperature (ºC)	K_2 autocatalytic ($M^{-1} min^{-1}$)	Slow initiation ($M^{-1} min^{-1}$)	Pre-equilibrium core ($M^{-1} min^{-1}$)
25	$4.7\pm0.1*10^{-11}$	$8.3\pm0.1*10^{-6}$	$7.8\pm0.1*10^{-7}$
50	$2.1\pm0.2*10^{-8}$	$4.9\pm0.1*10^{-5}$	$4.0\pm0.2*10^{-5}$
70	$1.6\pm0.2*10^{-7}$	$9.9\pm0.3*10^{-4}$	$3.1\pm0.1*10^{-5}$

Conclusion

The results of measuring the absorbance of the sample are indicative of an increase in reaction rate with increasing temperature. The results of fitting data in models show the mechanism of aggregation in retepelase protein solution has a high conformity with Autocatalytic (AC) model. With increasing temperature, following this process from Autocatalytic model increases. Given these results, it can be said that the formation of aggregate in reteplase protein solution will follow the mechanism of nucleation - growth. The low compliance of the data in the slow initiation model, in which the nucleation step is also removed, confirmed that the presence of nucleation stage in the reaction leading to the aggregate formation of the reteplase protein.Slow initiation and pre equilibrium core had the best fitting in n=2. This result will increase the formation of the core with connecting two protein monomers. Two protein monomers in an irreversible reaction adjoin together and produce cores. Also, the results of the rate of reaction constants show an increase in the reaction rate as the temperature increases. Near the melting point, increasing the rate of reaction was happen in a slower rate. $K_1 < K_2$ shows the result that limitation step of reaction is nucleation. The covalent bonds make aggregate, and it cannot be broken by reducing substances such as urea and sodium dodeyl sulfate.

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