Korean J. Chem. Eng., **30**(10), 1939-1945 (2013)
DOI: 10.1007/s11814-013-0143-3

Nucleation behavior of glutathione polymorphs in water

Zhi Chen, Leping Dang, Shuai Li, and Hongyuan Wei†

School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, P. R. China
(*Received 22 April 2013 • accepted 31 July 2013*)

Abstract−Nucleation behavior of glutathione (GSH) polymorphs in water was investigated by experimental method combined with classical nucleation theory. The solubility of α and β forms GSH in water at different temperatures, and the nucleation induction period at various supersaturations and temperatures were determined experimentally. The results show that, in a certain range of supersaturation, the nucleation of β form predominates at relatively higher temperature, while α form will be obtained at lower temperature. The nucleation kinetics parameters of α and β form were then calculated. To understand the crucial role of temperature on crystal forms, "hypothetic" nucleation parameters of β form at 283.15 K were deduced based on extrapolation method. The results show that the interfacial tension, critical free energy, critical nucleus radius and nucleus number of α form are smaller than that of β form in the same condition at 283.15 K, which implies that α form nucleates easier than β form at low temperature. This work may be useful for the control and optimization of GSH crystallization process in industry.

Key words: Glutathione, Nucleation, Induction Period, Crystallization, Classical Nucleation Theory

INTRODUCTION

Glutathione is a bioactive peptide, having important physiological functions of scavenging free radical, detoxification, promoting the absorption of iron, maintaining the integrity of the red cell membrane, DNA biosynthesis, the normal growth of cells and cellular immune variety [1]. Two kinds of glutathione exist in vivo: reduced glutathione (referred to as GSH) and oxidized glutathione (referred to as GSSG). The reduced glutathione abounds in the body and plays a major role [2,3].

Reduced glutathione is condensed by a peptide bond in L-glutamic acid, L-cysteine and glycine, known as γ-L-glutamyl-cysteinyl-glycine (short for GSH) [4]. Harington and Mead [5] first synthesized it in 1935 and confirmed its composition. Then it was verified by Vigneaud [6]. It is soluble in water, dilute alcohol, ammonia and dimethyl formamide, but insoluble in alcohol, ether and acetone [7]. Glutathione in solid state is more stable, but is unstable in aqueous solution [8].

GSH exists as two forms: α form and β form. The α form (see Fig. 1(a)) is colorless and needle-like with good solubility, and its crystal structure is called "the folded form," while the β form (see Fig. 1(b)) is colorless and fine with bad solubility and its structure is called "the extended form" [9]. Element analysis, paper electrophoresis, specific optical rotation, melting point and NMR of α form quite agree with those of β form. Infrared spectroscopy and XRD analysis can be used to distinguish the two forms of GSH. In the

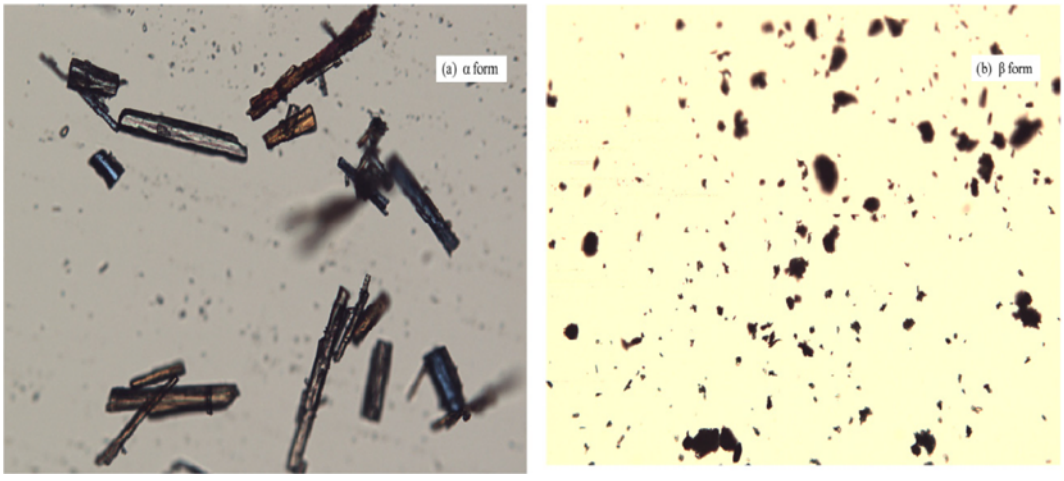

Fig. 1. Microscope pictures of α and β form GSH (Magnification 100×).

†To whom correspondence should be addressed.
E-mail: crystallization.wei@hotmail.com

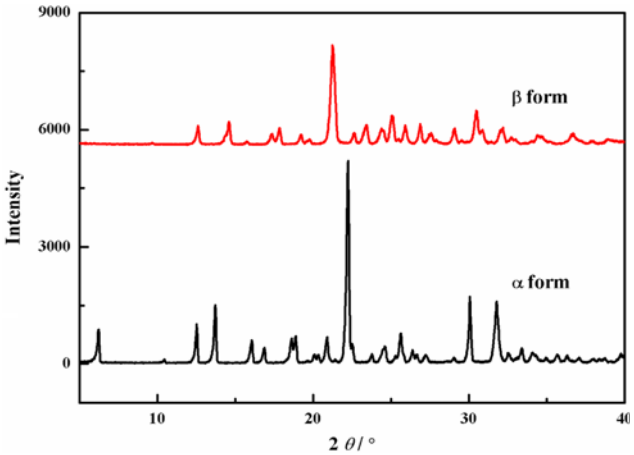

Fig. 2. XRD patterns of α form and β form GSH.

XRD patterns (see Fig. 2), the α form shows a very strong characteristic peak at d=4.00 Å, while β form at d=4.19 Å [9].

As for the advantage of good solubility, α form GSH becomes the desired crystal form in the pharmaceutical industry. However, very strict control is needed during crystallization since trace amounts of β form crystal will affect the quality of the products, e.g., affect clarity of solution. Therefore, how to control the nucleation of the two forms in solution becomes a vital issue that requires to inhibit the nucleation of β form and promote α form nucleation. At present, studies on glutathione focus on its production and detection method. Its nucleation behavior has not been systematically reported yet. We obtained the important nucleation properties, including solid-liquid interfacial tension, critical nucleation free energy and nucleus radius, by analyzing the primary nucleation process. The results highlight the nucleation behavior of GSH polymorphs in water, and may be useful in guiding industrial crystallization processes.

THEORETICAL BASIS

Nucleation is an extremely important research topic in the field of industrial crystallization. The nucleation process can be divided into two categories: primary nucleation and secondary nucleation [10]. Due to the limitations of research means in the microscopic field, study on primary nucleation is still quite difficult. In general, the determination of the induction period can establish a link between the classical nucleation theory and experimental study, and thus becomes an important means to study the nucleation behavior.

1. Induction Period

In the crystallization process, the crystal nucleus does not appear immediately when supersaturation is obtained. Its formation needs to go through a so-called induction period. The induction period is referred to as the time required for "first nucleation events" to be detected in the solution kept at a constant level of supersaturation [11].

2. Homogeneous Nucleation

According to the classical primary nucleation theory [10], the rate equation for homogeneous nucleation can be expressed as

$$J = A\exp\left[-\frac{16\pi\gamma^3 v^2}{3k^3T^3\ln^2 S}\right] \quad (1)$$

where J is the rate of nucleation, A is the pre-exponential factor, S is the supersaturation ratio, γ is the interfacial tension, v is the molecular volume, k is the Boltzmann constant, T is the thermodynamic temperature.

EXPERIMENTAL SECTION

1. Materials

Glutathione (over 98% mass fraction purity) in experimental work was provided by Hisun Pharmaceutical Co., Ltd., China. The distilled water employed in this work was purchased commercially from Tianjin Kewei Co.of China.

2. Solubility Measurement

The solubility was measured by the gravimetric method [12]. Slurry was prepared by adding excess glutathione into water in glass vessels, stirring for a period of time in a constant temperature water bath. After achieving solid-liquid equilibrium, the suspension was allowed to stand for layering. The supernatant was extracted by injector (membrane filtration, 0.2 μm), and the sample was weighted after drying by using an analytical balance (FA2004, Shanghai Shanping Yiqi Co. Ltd., China) with an accuracy of ±0.1 mg. When the mass was unchanged, the solubility could be calculated. The undissolved solid was analyzed by powder X-ray diffraction (XRD) (Rigaku D/max-2500, Rigaku Corporation, Japan). Each experiment was repeated three times. The mole fraction solubility x_A of solute A in the solvent B can be calculated from the following formula:

$$x_A = \frac{m_A/M_A}{m_A/M_A + m_B/M_B} \quad (2)$$

where x_A is the mole fraction solubility, m_A and m_B represent the masses of the solute and solvent and M_A and M_B represent the molecular weights of the solute and solvent, respectively.

3. Induction Period Measurement

The induction period of glutathione solution was measured by means of the laser light scattering method [13]. The test apparatus for the determination of induction period is shown in Fig. 3. The laser monitoring system (HNK250, Beijing University Physics Department Factory, China) consists of a laser generator, a photoelectric transformer and a recorder. A certain amount of glutathione and water was added into a 40 mL glass jacketed crystallizer with continuous stirring and heated to dissolve all the solute. The laser monitoring system was turned on. The solution was then rapidly cooled to the desired temperature. Thus, the supersaturated solution was

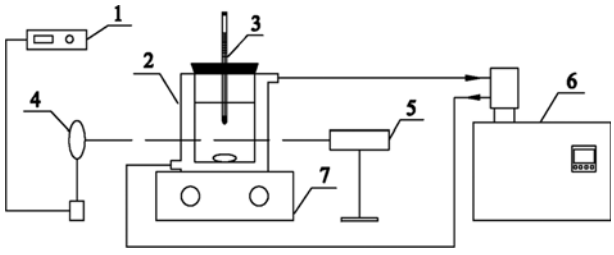

Fig. 3. The experimental device for induction period measurement.

1. Recorder
2. Jacketed crystallizer
3. Thermometer
4. Photoelectric transformer
5. Laser generator
6. Thermostatic water bath
7. Magnetic stirrer

obtained. As nucleation began, the intensity of the laser beam penetrating the vessel dramatically dropped to a minimum. The corresponding time was recorded, and the induction time was regarded as the time interval from the attainment of supersaturation until the abrupt decrease in the laser intensity profile occurred. Solution was instantly filtered after the crystals precipitated. The crystals were dried and the polymorphic form was identified by XRD. Changing the supersaturation ratio of the solution and the temperature to repeat the procedure above, the induction periods can be obtained under different conditions. Every experiment was repeated three times and the average value was used as the induction time. Estimated uncertainties of the experimental values were less than 5% calculated from the relative standard deviation.

The supersaturation ratio S=C/C*, where C is the initial concentration of the solution, and C* is the equilibrium solubility of given form GSH at the investigated temperature. Since glutathione aqueous solution is unstable and easily decomposed at high temperature, the highest concentration of glutathione in aqueous solution achieved in this work is below 0.03118 (mole fraction). Then a higher supersaturation can only be achieved by reducing the temperature.

RESULTS AND DISCUSSION

1. Solubility of and Form GSH

The solubility of two forms of glutathione in water is shown in Fig. 4. Solubility data was fitted by Apelblat equation [14]. It can be found that the solubility of two forms increases with increasing temperature. At the same temperature, the solubility of α form is obviously greater than β form. The α form is the metastable form in aqueous solution.

2. Effect of Supersaturation and Temperature on Induction Period

The induction period of glutathione in water was measured from 283.15 K to 308.15 K; the relationship between induction period and supersaturation is shown in Fig. 5. As can be seen from Fig. 5, the induction period decreases exponentially with increasing supersaturation, which suggests that the nucleation rate increases exponentially with increasing supersaturation. As the temperature increases, the induction period is greatly reduced at the same level of supersaturation. In addition, Fig. 5 also shows that the induction period significantly decreases with the increase of supersaturation within higher supersaturation range. However, within lower supersaturation range, the degree of decline in the induction period becomes smaller. Such different trends may be attributed to the change of the nucleation kinetics resulting from the nucleation of polymorphs.

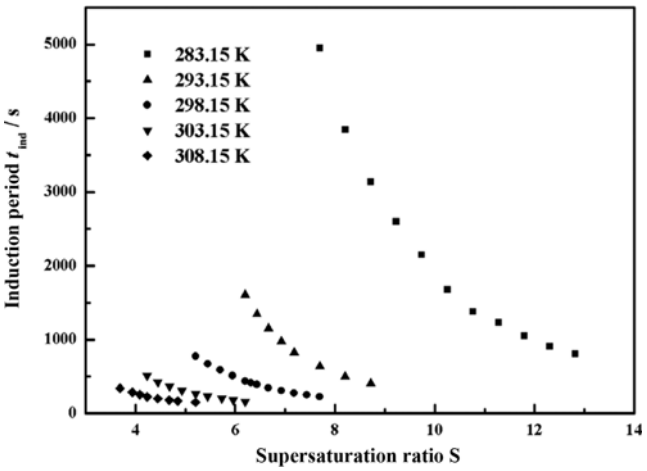

Fig. 5. Effect of supersaturation ratio and saturation temperature on the induction period.

3. Effect of Temperature on the Crystal Forms

To investigate the influence of supersaturation and temperature on the crystal form, crystals obtained at different supersaturation were analyzed at a given temperature by XRD. It turns out that the crystals are β form at 293.15 K, 298.15 K, 303.15 K and 308.15 K in the measured range of supersaturation. The XRD results at 293.15 K, as a typical case, are shown in Fig. 6. However, at 283.15 K, the crystal form is α form as shown in Fig. 7 when S is between 7.7 and 12.8. Therefore, temperature can be a key factor that determines the crystal forms during the nucleation of GSH in water. Higher temperature promotes nucleation of β form GSH, while lower temperature is favored for obtaining form crystals.

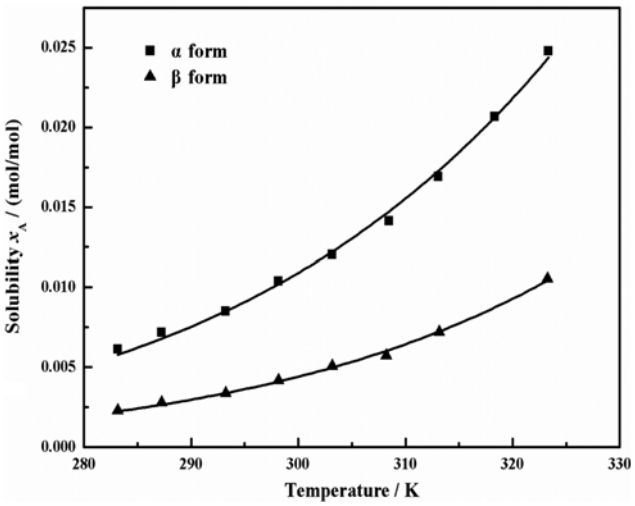

Fig. 4. The solubility curve of α form and β form GSH in water.

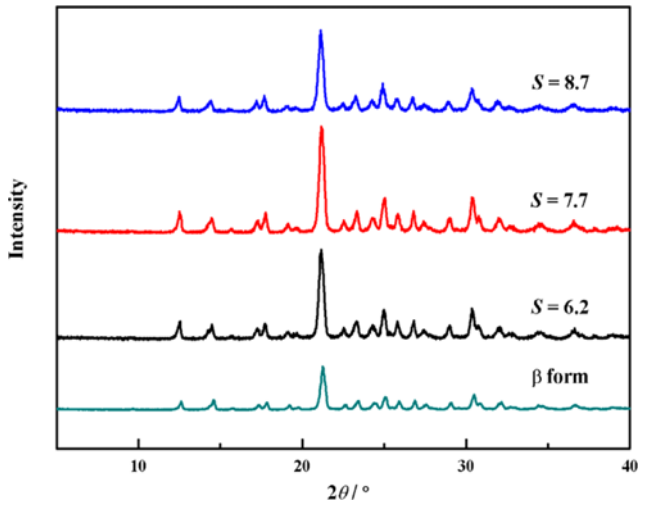

Fig. 6. XRD patterns of β form nuclei at 293.15 K.

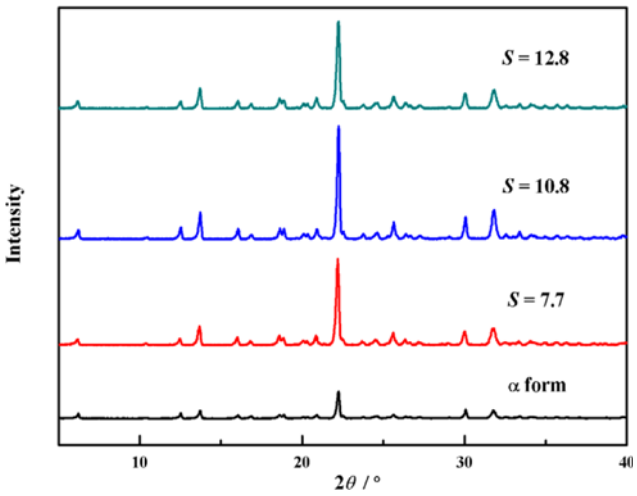

Fig. 7. XRD patterns of α form nuclei at 283.15 K.

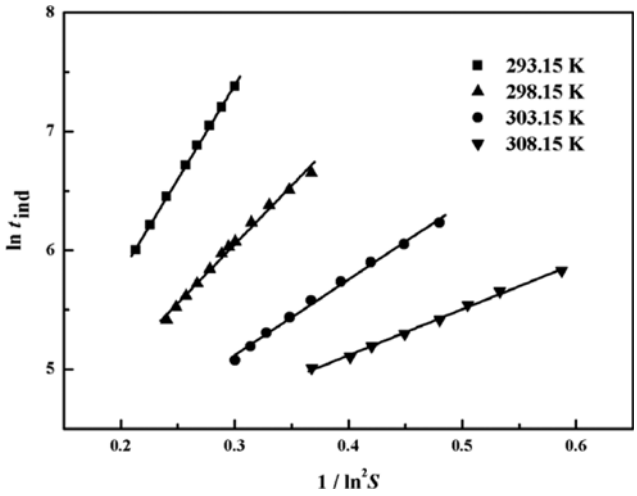

Fig. 8. Plot between ln t_{ind} and 1/ln²S for β form at different temperatures.

4. Interfacial Tension

The critical parameter involved between a growing crystal and the surrounding mother liquor is the interfacial tension [15]. Crystal-solution interfacial tension is a crucial parameter involved in the theories of crystal nucleation and growth, which determines the mechanism of crystal growth to a great extent. In the present investigation, interfacial tension can be calculated by using the experimentally determined induction period values on the basis of classical theory for homogeneous formation of spherical nuclei [16].

For homogeneous nucleation, the nucleation rate is inversely proportional to the induction period:

$$t_{ind}^{-1} \propto J \tag{3}$$

From Eqs. (1) and (2), the relationship between induction period and supersaturation can be expressed as

$$\ln t_{ind} = B + \frac{16\pi\gamma^3 v^2}{3k^3T^3\ln^2 S} \tag{4}$$

At constant temperature, a straight line for ln t_{ind} against 1/ln²S should be obtained. The slope of this straight line can be given by

$$l = \frac{16\pi\gamma^3 v^2}{3k^3T^3} \tag{5}$$

Therefore, the solid-liquid interfacial tension can be calculated from

$$\gamma = \left[\frac{3lk^3T^3}{16\pi v^2}\right]^{1/3} \tag{6}$$

Ramsay-Shields [17] suggested that the relationship between the interfacial tension and the temperature can be expressed as follows:

$$\gamma V^{2/3} = K(T_c - T - 6) \tag{7}$$

Where, V is the molar volume, T_c is the critical temperature, K is a constant.

According to Eq. (7), the plot of γ against T is a straight line in general.

In the following work, crystal-solution interfacial tension of β form and α form was calculated to understand the nucleation behavior of glutathione in water. As seen from Fig. 5, the induction period depends much on the initial supersaturation. When the initial supersaturation is high enough, it is considered to be homogeneous nucleation. At 293.15 K, 298.15 K, 303.15 K and 308.15 K, the plot of ln t_{ind} against 1/ln²S for β form is shown in Fig. 8. Then, the solid-liquid interfacial tension of glutathione β form at various temperatures can be obtained by Eq. (6), as shown in Table 1.

According to Eq. (7), the interfacial tension is a linear function of the temperature and is plotted in Fig. 9. The fitted linear equation is expressed as

Table 1. Interfacial tension values for β form at different temperatures

Temperature T/K	Slope l	Interfacial tension γ/(mJ/m²)	R^2
293.15	15.81979	8.03222	0.99952
298.15	9.88138	6.98317	0.99246
303.15	6.37330	6.13467	0.99472
308.15	3.87553	5.28305	0.99590

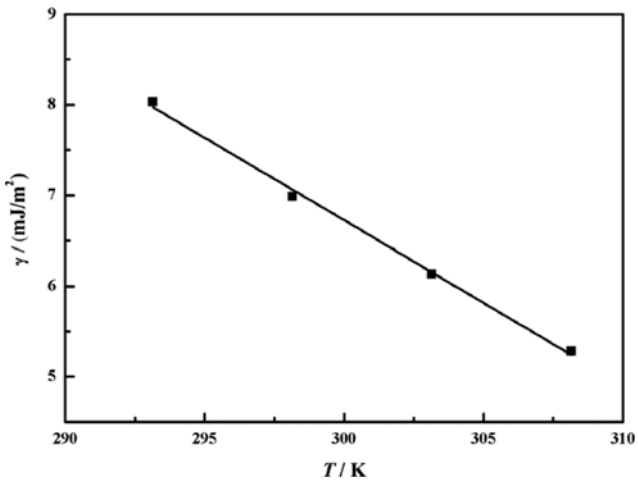

Fig. 9. The interfacial tension γ as a function of temperature T for β form.

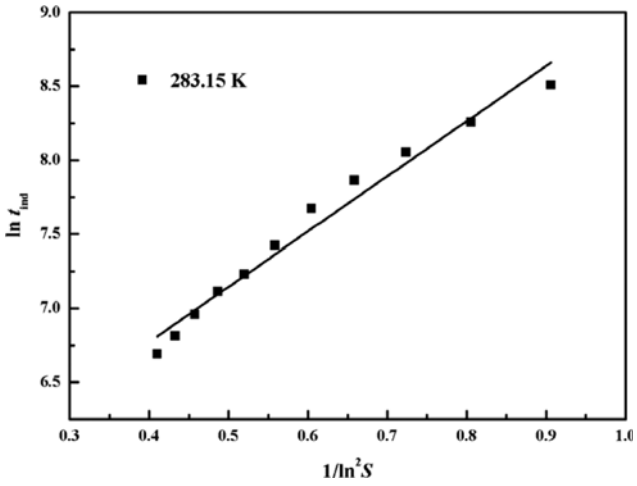

Fig. 10. The plot of ln t_{ind} against $1/\ln^2 S$ for α form at 283.15 K.

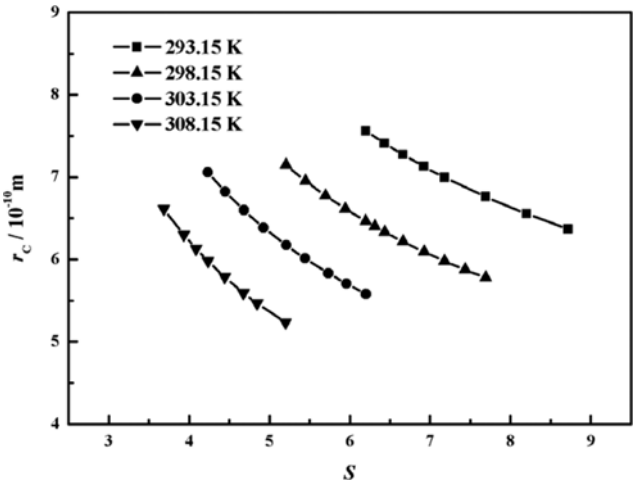

Fig. 11. Relation between critical nucleus radius (r_c) and supersaturation for β form GSH.

$$\gamma = 61.30259 - 0.18192T \quad (8)$$

The regression coefficient R^2 is 0.99573. As can be seen from the above work, α form crystal will be obtained rather than β form when glutathione nucleates in water under a lower temperature such as 283.15 K. To reveal the difference of nucleation parameters between α form and β form GSH, extrapolation was used to obtain a "hypothetic" interfacial tension of β form at 283.15 K. Hence, the interfacial tension value γ of β form at 283.15 K can be calculated by Eq. (8), which is 9.79194 mJ/m^2.

At 283.15 K, the plot of ln t_{ind} against $1/\ln^2 S$ for α form is shown in Fig. 10. The slope of the fitted line is 3.72862 and the intercept is 5.28155. The regression coefficient R^2 is 0.97286. Therefore, the interfacial tension of glutathione α form at 283.15 K is obtained by using Eq. (6). The interfacial tension value γ for α form is 4.79231 mJ/m^2 at 283.15 K, which is smaller than that of β form compared with the calculated value above.

5. Critical Nucleation Parameters

The critical nucleus radius is an important parameter in the nucleation process. Only the nuclei whose radius is larger than the critical nucleus radius can exist stably and continue to grow.

According to the classical nucleation theory derived from the work of Gibbs, the change in the Gibbs free energy (ΔG) required to form nuclei is given by [18]

$$\Delta G = \Delta G_S + \Delta G_V = 4\pi r^2 \gamma + \frac{4}{3}\pi r^3 \Delta G_v \quad (9)$$

$$-\Delta G_v = \frac{kT \ln S}{v} \quad (10)$$

Where, ΔG_s is the energy change per unit surface, ΔG_v is the energy change per unit volume, r is the radius of the nucleus, γ is the interfacial tension.

At the critical state, the free energy formation obeys the condition d(ΔG)/dr=0. There exists a critical free energy ΔG_c corresponding to the critical nucleus radius r_c.

The critical nucleus radius is

$$r_c = \frac{2v\gamma}{kT \ln S} \quad (11)$$

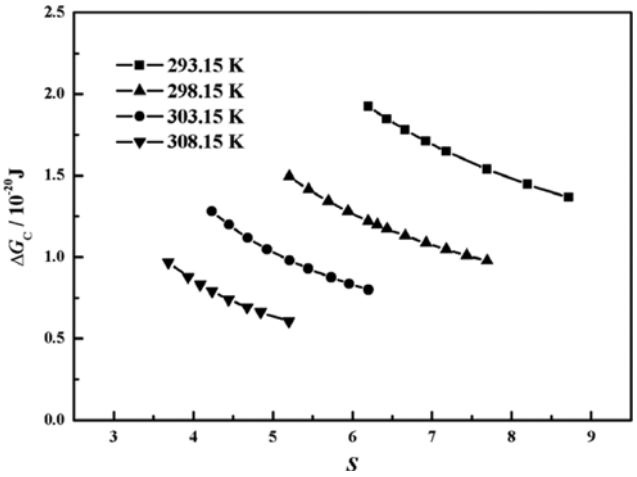

Fig. 12. Relation between critical free energy (ΔG_c) and supersaturation (S) for β form GSH.

The critical free energy barrier is

$$\Delta G_c = \frac{16\pi v^2 \gamma^3}{3k^2 T^2 \ln^2 S} \quad (12)$$

The molecular number of critical nuclei is expressed as

$$N_c = \frac{32\pi\gamma^3 v^2}{3k^3 T^3 \ln^3 S} \quad (13)$$

According to Eqs. (11) and (12), the critical nucleus radius (r_c) and the critical free energy (ΔG_c) of β form for various temperatures are obtained and shown in Fig. 11 and Fig. 12. It is clear that the critical nucleus radius and the critical free energy apparently decrease with the increase of supersaturation at the same temperature. The same trend prevails for the number of the critical nucleus. Moreover, as temperature increases, there is a marked drop in the critical nucleus radius and the free energy at the same level of supersaturation. The higher the temperature, the greater the decline. It is consequently interpreted that the enhancement of temperature or supersaturation makes the nucleation easier when one parameter is fixed.

By calculating γ, the free energy change for α and β form in dif-

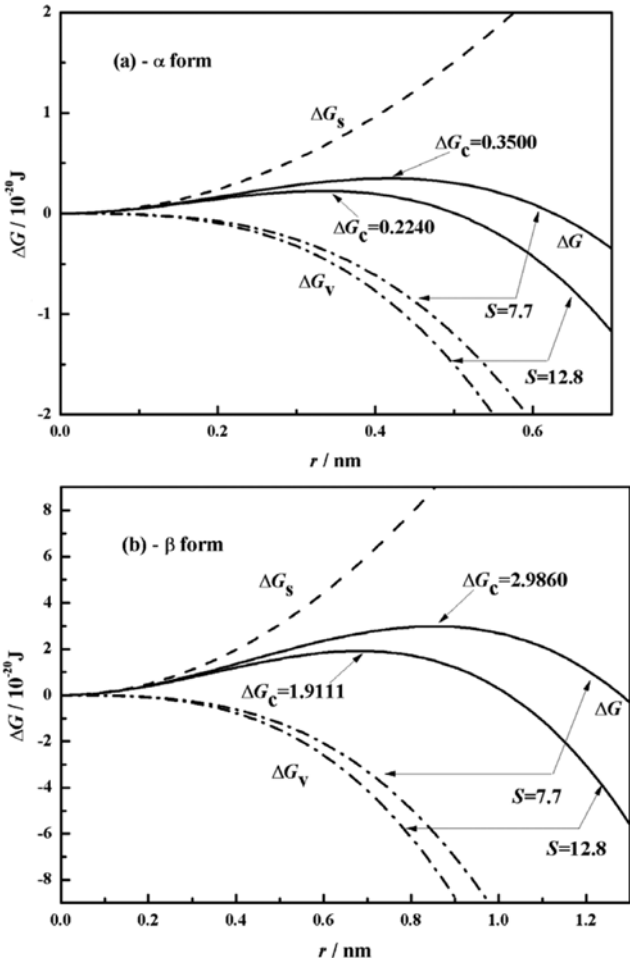

Fig. 13. Free energy change (ΔG) graph for the nucleation of GSH at 283.15 K, ΔG_v is the free energy change per unit volume, ΔG_s is the free energy change per unit surface, r is the crystal nucleus radius, and S is the supersaturation ratio.

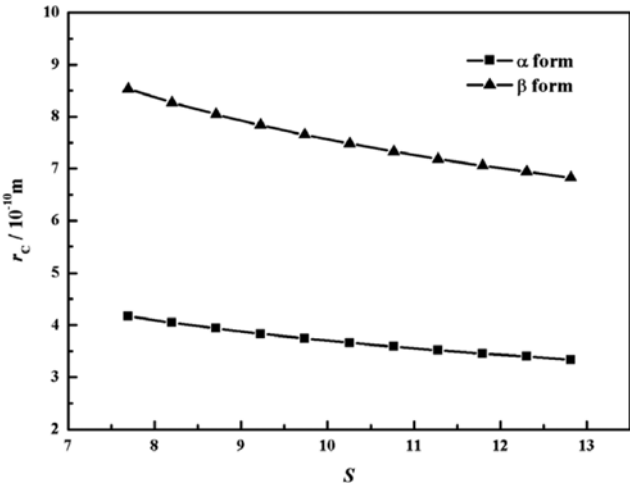

Fig. 14. Critical nucleus radius (r_c) of α form and β form GSH against supersaturation (S) at 283.15 K.

ferent supersaturation levels at 283.15 K can be obtained from Eqs. (9) and (10), as graphically shown in Fig. 13(a) and Fig. 13(b). The critical radius r_c, critical free energy ΔG_c and nucleus number N_c

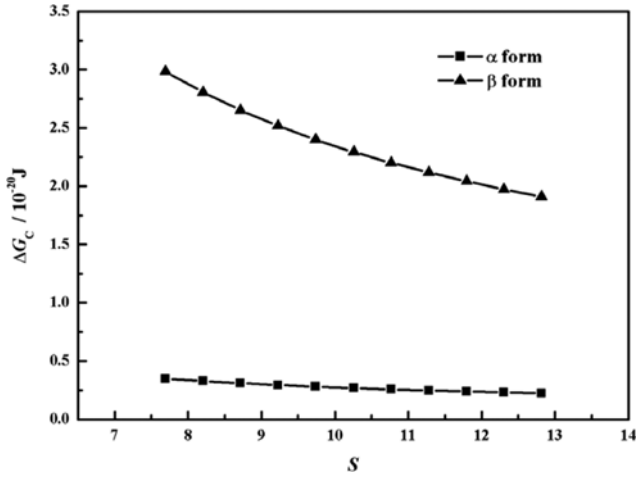

Fig. 15. Critical free energy (ΔG_c) of α form and β form GSH against supersaturation (S) at 283.15 K.

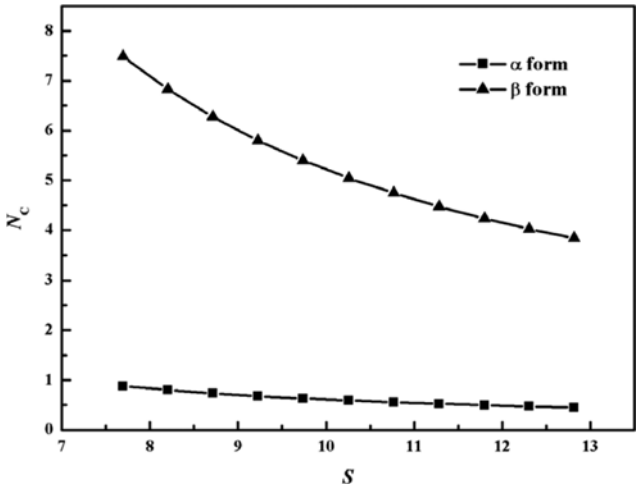

Fig. 16. Critical nucleus number (N_c) of α form and β form GSH against supersaturation (S) at 283.15 K.

for α form and β form at 283.15 K are evaluated using Eqs. (11)-(13). The evaluated outcomes as a function of supersaturation are depicted in Figs. 14, 15 and 16, respectively.

Compared with β form (Fig. 13(b)), r_c and ΔG_c of α form are significantly smaller under the same conditions of temperature (283.15 K) and supersaturation (Fig. 13(a)), indicating the presence of a smaller energy resistance to form α crystal nuclei. In contrast, it also can be found that the critical nucleus number N_c of α form is less than that of β form. It is consequently interpreted that α form GSH nucleates easier than β form under these conditions. Therefore, in order to obtain α form of GSH in industry, crystallization process should be controlled at a certain lower temperature.

CONCLUSIONS

Nucleation behavior of GSH polymorphs in water was systematically investigated based on the classical nucleation theory. The conclusions are as follows.

First, the induction period of two forms decreases with the increase

of supersaturation or temperature. β form crystals generate at relatively high temperature, while form crystals form at low temperature (283.15 K) in the measured range of supersaturation.

Secondly, "hypothetic" nucleation parameters of β form at 283.15 K were calculated by using extrapolation method. By comparison, the interfacial tension value γ for α form at 283.15 K is smaller than the calculated value of β form. Also, the critical free energy, critical nucleus radius and nucleus number of α form are much smaller than that of β form under the same conditions of temperature and supersaturation, which means that it has a lower energy resistance to form α crystal nuclei; thus α form of GSH nucleates easier than β form at low temperature. Hence, in order to obtain α form glutathione in industry, the crystallization process should be controlled at a relatively low temperature.

ACKNOWLEDGEMENTS

We acknowledge the Doctoral Fund of Ministry of Education of China (20110032110022), and Tianjin Municipal Natural Science Foundation (11JCYBJC 04600, 13JCQNJC05200) for their financial assistance in this project.

NOMENCLATURE

A : pre-exponential factor
C : the initial concentration of the solution
C* : the equilibrium solubility of β form GSH
J : the rate of nucleation
k : Boltzmann constant
K : a constant
l : slope of straight line
m_A, m_B : the masses of solute and solvent
M_A, M_B : molecular weights of solute and solvent
N_c : molecular number of critical nuclei
r : radius of the nucleus
r_c : critical nucleus radius
S : supersaturation ratio
T : thermodynamic temperature
T_c : critical temperature
t_{ind} : induction period
v : molecular volume
V : molar volume
x_A : mole fraction solubility of solute
γ : interfacial tension
ΔG : Gibbs free energy
ΔG_c : critical free energy
ΔG_s : the energy change per unit surface
ΔG_v : the energy change per unit volume

REFERENCES

1. J. Vina, *Glutathione: Metabolism and physiological functions*, CRC, Boca Raton, FL (1990).
2. M. Penninckx, *Enzyme Microb. Technol*., **26**, 737 (2000).
3. A. Meister and M. E. Anderson, *Ann. Rev. Biochem*., **52**, 711 (1983).
4. S. C. Lu, *Fed. Am. Soc. Exp. Biol. J.*, **13**, 1169 (1999).
5. C. H. Harington and T. H. Mead, *Biochem*., **29**, 1602 (1935).
6. V. D. Vigneaud and H. M. Dyer, *BioChem*., **5**, 159 (1936).
7. W. Li, S. S. Bollecker and J. D. Schofield, *J. Cereal Sci*., **39**, 205 (2004).
8. M. Aruga, S. Awazu and M. Hanano, *Chem. Pharm. Bull*., **26**, 2081 (1978).
9. M. Miyoshi, K. Kotera, H. Seko, K. Masukawa, S. Imado and K. Okumura, *Bull. Chem. Soc*., **42**, 1749 (1969).
10. J. W. Mullin, *Crystallization*, 4th Ed., Butterworth-Heinemann, Oxford (2001).
11. N. A. Mitchell, P. J. Frawley and C. T. Ó'Ciardhá, *J. Cryst. Growth*, **321**, 91 (2011).
12. A. Shalmashi and A. Eliassi, *J. Chem. Eng. Data*, **53**, 199 (2008).
13. Van A. E. S. Driessche, F. Otálora, G. Sazaki, M. Sleutel, K. Tsukamoto and J. A. Gavira, *Cryst. Growth Des*., **12**, 4316 (2008).
14. A. Apelblat and E. Manzurola, *J. Chem. Thermodyn*., **31**, 85 (1999).
15. K. Selvaraju, R. Valluvan and S. Kumararaman, *Mater. Lett*., **60**, 1565 (2006).
16. M. Z. Volmer, *Phys. Chem*., **119**, 277 (1926).
17. W. Ramsay and J. Shields, *J. Chem. Soc., Trans.*, **63**, 1089 (1893).
18. A. E. Nielsen and S. Sarig, *J. Cryst. Growth*, **8**, 1 (1971).