Lait 87 (2007) 139–148
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DOI: 10.1051/lait:2007005

Available online at:
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Original article

Kinetic and thermodynamic studies on the thermal denaturation of bovine milk insulin-like growth factor-I in model systems

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Received 21 November 2006 – Accepted 30 January 2007

Abstract – In this study, kinetic and thermodynamic parameters for heat denaturation of bovine milk insulin-like growth factor-I (IGF-I) in phosphate-buffered saline (PBS) and ultra-high temperature (UHT) milk were determined. The D-values were higher in UHT milk than in PBS at all temperatures. The Z-values were 24.41 °C and 27.12 °C in PBS and UHT milk, respectively. Heat denaturation of bovine milk IGF-I followed a reaction order of n = 1.1. The energies of activation and change in Gibbs free energy, enthalpy and entropy were also compared. The results showed more significant thermal stability of IGF-I in milk than in PBS.

kinetics / thermodynamics / heat denaturation / milk / insulin-like growth factor-I

摘要 – 牛乳类胰岛素生长因子 -I 在模型系统中的热变性动力学和热力学研究。本文研究并测定了牛乳类胰岛素生长因子 -I (IGF-I) 在两个模型系统 --- 磷酸盐缓冲液 (PBS) 和 UHT 乳中的热变性动力学和热力学参数。计算结果表明，在选定的加热温度下， IGF-I 在 UHT 乳中的热变性 D- 值均高于在 PBS 中的 D- 值; 在 PBS 和 UHT 乳中的热变性 Z- 值分别为 24.41 °C 和 27.12 °C; 且牛乳 IGF-I 的热变性反应级数为 1.1 级。同时对 IGF-I 在两个系统中的活化能、吉布斯自由能变、焓变和熵变进行了比较。结果显示：牛乳 IGF-I 在 UHT 乳中有更强的热稳定性。

动力学 / 热力学 / 热变性 / 牛乳 / 类胰岛素生长因子 -I

Résumé – Étude cinétique et thermodynamique de la dénaturation thermique du facteur de croissance IGF-I du lait bovin dans des systèmes modèles. Dans cette étude, les paramètres cinétiques et thermodynamiques de la dénaturation thermique du facteur de croissance IGF-I ont été déterminés dans une solution de tampon phosphate (PBS) et dans un lait traité à ultra-haute température (UHT). Les valeurs D étaient plus élevées pour le lait UHT que pour le milieu PBS pour toutes les températures. Les valeurs Z correspondaient à 24,41 °C et 27,12 °C dans le milieu PBS et le lait UHT, respectivement. La dénaturation thermique du facteur IGF-I bovin suivait une réaction d'ordre n = 1,1. Les énergies d'activation et la variation de l'énergie libre de Gibbs, de l'enthalpie et de l'entropie ont également été comparées. Les résultats montrent que la stabilité thermique du facteur IGF-I était significativement plus élevée dans le lait par rapport au milieu PBS.

cinétique / thermodynamique / dénaturation thermique / lait / facteur de croissance IGF-I

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1. INTRODUCTION

Colostrum is a complex fluid rich in lots of bioactive components, including many growth factors, especially insulin-like growth factor-I (IGF-I). This compound is a 70-amino acid globular protein with a molecular mass of 7650 g·mol^{-1} and isoelectric point of 8.2 [32]. The primary structure of IGF-I is a single-chain polypeptide containing three intramolecular disulfide bonds. A similar amino acid sequence was identified in human and bovine IGF-I [15]. IGF-I is present in almost all mammalian milk and abundant in bovine colostrum, but the concentration in bovine milk shows wide variation, from 50~2950 ng·mL^{-1} [35].

IGF-I comprises the major and principal milk-based growth factors and exerts extensive fundamentally physiological functions, such as promoting protein and DNA synthesis, stimulating cell differentiation and proliferation, and modulating growth and development of the newborn animal's gastrointestinal tracts. Burrin et al. [4] reported that orally administered IGF-I improved intestinal mucosal growth and ileal protein in formula-fed neonatal pigs. A similar effect has been found to increase small intestine weights, duodenal crypt depths and villi heights in weaned rats [13] and to enhance the intestinal villus size [27]. IGF-I has also been demonstrated to promote cell growth in cultured mammary tissues in vitro [31, 37] and DNA synthesis in piglet intestines in vivo [17]. In addition, milk-derived IGF-I has been applied to replace serum in different human cell cultures [2], to stimulate wound repair [26] and to attenuate chemotherapeutic drug toxicity in an animal model [5, 18, 33] as therapeutic agents. In fact, IGF-I also shows promise as a nutraceutical supplement in the food industry, and in clinical and pharmaceutical development.

Heat treatment is a basic and extensive unit processing, and knowledge concerning the effects of heating on IGF-I bioactivity is very important and essential. Although several studies have investigated the thermal stability of bovine IGF-I in milk at a single temperature and time [6, 10, 11, 19], no relative and comprehensive study on the kinetic and thermodynamic parameters for thermal denaturation of bovine milk IGF-I has been reported.

The object of this investigation was to determine the thermal stability of bovine milk IGF-I in two model systems of phosphate-buffered saline and UHT milk, to calculate the kinetic and thermodynamic parameters for the denaturation process, in order to predict the recovery in different temperature-time courses and to facilitate the design of heat treatment that preserves its biological activity.

2. MATERIALS AND METHODS

2.1. Milk supply

Colostrums were obtained from healthy Holstein cows from Inner Mongolian Agriculture University teaching farms. Colostrum samples were collected within 24 h postpartum, and immediately skimmed by centrifugation at 3000× *g* for 30 min at 4 °C. The skimmed colostrum samples were frozen and stored at –40 °C. Commercial UHT milk with 3.3% fat and 2.9% protein, produced by heating at 137 °C for 4 s, was purchased from local supermarkets. Sephadex G-15 chromatograph gel and all other reagents were supplied by Sigma Chemical Co. (St Louis, MO, USA).

2.2. Preparation of crude IGF-I

The preparation of crude IGF-I was performed by the method of Francis et al. with some modifications [15]. Specifically, 100 mL of skimmed colostrum were added to 400 mL of HCl (2 mol·L^{-1})-ethanol (100%) solution (1:7, vol/vol), and were blended vigorously using an IKA RW20 blender (JANK & KUNKEL CO, STAUFEN, Germany) at 4 °C for 30 min. The precipitation was removed from the mixture by centrifugation at 3500× *g* for 30 min at 4 °C and the supernatant was adjusted to pH 6.4 using 5 mol·L^{-1} NaOH. The neutral supernatant was then concentrated to around 50 mL at 20 °C and a pressure < 10 μbar using a N-N series Rotary Vacuum Evaporator (RIKAKIKAI Co., LTD, Tokyo, Japan). After centrifugation at 3500× *g* for 20 min at 4 °C, 25 mL of the supernatant was chromatographed through

a Sephadex G-15 column (90 × 3 cm) and eluted with demineralized water. The first peak fraction, which contained the maximum IGF-I concentration, was freeze-dried at –40 °C and a pressure < 100 μbar by the LABconco FreeZONE 2.5 Freeze Dry system (Labconco Corporation, Kansas, USA) and the lyophilized powder, in which IGF-I concentration was 2.013 $mg \cdot g^{-1}$, was used as a source of crude IGF-I.

2.3. Heat treatment of bovine IGF-I in two model systems

Crude IGF-I was dissolved in pH 6.60 PBS (0.01 $mol \cdot L^{-1}$ phosphate buffer and 0.85% NaCl saline) or in commercial UHT milk, pH 6.64. The final IGF-I concentration was 61.166 $ng \cdot mL^{-1}$ and 61.091 $ng \cdot mL^{-1}$ in PBS and UHT milk, respectively. Previous experiments had indicated that the concentration of IGF-I was below the kit detection limit of 1 $ng \cdot mL^{-1}$ in UHT milk, which may be due to the dilution effect of extraction and neutralization procedures in the RIA assay.

Five hundred μL of IGF-I solution were placed in thin-wall (0.6 mm) glass test tubes (40 × 8 mm) and covered with Parafilm. The tubes were incubated in a SB-651 Digital oil baths system at 65, 72, 80 or 90 °C. Six individual test tubes were removed from the oil bath at the specified holding time and rapidly cooled in ice water. The time taken for the solution to reach the equilibrated temperature of the oil baths ranged between 15 and 25 s.

2.4. Determination of immunoreactive IGF-I concentration

The immunoreactive IGF-I concentrations in the samples were determined by a solid-phase "sandwich" radio immunoassay (RIA) using an IGF-I test kit (CIS BIO International, France). Two monoclonal antibodies were prepared against two different antigenic sites of IGF-I molecules. The first was coated on the tube, and the second, radio-labeled with iodine 125, was used as a tracer. The pretreatment of samples was performed by the methods of Etherton et al. [12]. Briefly, 200 μL of milk samples were extracted with 800 μL acid/ethanol solution. After removal of protein precipitation through centrifugation, 500 μL supernatant were neutralized with 200 μL 0.855 $mol \cdot L^{-1}$ Tris and the neutralized extract were used for the RIA. All RIAs accorded strictly with the assay procedure and protocol of the test kit, and the radioactivity was counted with a LKB 1218 gamma counter (LKB-Wallac, TURKU10, Finland) for 1 min. All determinations were performed in duplicate.

2.5. Parameter calculation and statistical analyses

2.5.1. D- and Z-values

The D-values (time for 90% IGF-I denaturation at constant temperature) were calculated for each heating temperature by the reciprocal slope of the line from plotting the logarithm of IGF-I concentration versus heating time. The Z-value (temperature necessary to reduce D-value in one logarithmic cycle in the range of temperature) was obtained by the negative reciprocal slope of the line from plotting the logarithm of D-value versus heating temperature.

2.5.2. Reaction order and rate constant

The orders of reaction and rate constant for denaturation of IGF-I were determined by the general rate equation:

$$-(dc/dt) = k \cdot C^n \quad (1)$$

where –(dc/dt) represents the rate of IGF-I denaturation, k the rate constant, C the IGF-I concentration at each time, and n the reaction order.

For n = 1: integrating the general rate Equation (1),

$$\ln(C_t/C_0) = -k \cdot t \quad (2)$$

where C_0 represents initial IGF-I concentration and C_t the concentration of undenatured IGF-I at each holding time.

The value of the constant, k, is obtained from the slope of the straight line by regression analysis.

For $n \neq 1$: integrating the general rate Equation (1),

$$(C_t/C_0)^{1-n} = 1-(n-1)\cdot k\cdot C_0^{n-1}\cdot t = 1-(n-1)\cdot k'\cdot t$$

$$(\text{where } k' = k\cdot C_0^{n-1}). \quad (3)$$

The presentation of Equation (3) yields a straight line, and from its slope the k' value is obtained. The true value of k can be calculated from the expression $k' = k\cdot C_0^{n-1}$. The ordinate intercept b should be b = 1 for a non-first-order reaction.

2.5.3. Kinetic and thermodynamic parameters

The kinetic parameter was obtained from the Arrhenius equation:

$$\ln k = \ln A - (Ea/R)\cdot(1/T) \quad (4)$$

where A represents the Arrhenius constant, k the rate constant, Ea energy of activation, R the universal gas constant and T the absolute temperature. When ln k is plotted against 1/T according to Equation (4), the Ea value from the slope and the ln A value from the ordinate intercept are obtained. The thermodynamic parameters ΔH (change in enthalpy), ΔS (change in entropy) and ΔG (change in Gibbs free energy) are obtained by the following equations:

$$\Delta H = Ea - R\cdot T$$

$$\Delta S = R\cdot(\ln A - \ln (K_B/h_p) - \ln T)$$

$$\Delta G = \Delta H - T\cdot\Delta S$$

where K_B represents the Boltzmann constant and h_p the Planck constant.

2.6. Statistical analyses

Least squares linear regression analyses of the data on all plots were performed using SAS 6.03 [30]. In all plots, the models obtained by linear regression analysis were highly significant ($P < 0.001$).

3. RESULTS

3.1. Thermal denaturation of bovine milk IGF-I in two systems

The rate of denaturation of IGF-I and logarithmic plots of the IGF-I content in PBS and UHT milk as a function of heating time at 65, 72, 80 and 90 °C are shown in Figure 1 and Figure 2, respectively. The IGF-I concentration decreased with time and temperature of treatment. In all temperature-time courses, the denaturation rate of IGF-I was higher in PBS than in UHT milk. Table I shows the D- and Z-values for denaturation of IGF-I. Higher D-values were found in UHT milk for all temperatures, compared with PBS. The Z-values calculated by logarithmic plots of the D-values versus temperature (Fig. 3) were 27.12 °C in UHT milk, and 24.41 °C in PBS. This indicates a higher thermoresistance of IGF-I in UHT milk than in PBS over the temperature range studied.

Table I. D-values (s) and Z-values (°C) for thermal denaturation of crude IGF-I in PBS and UHT milk.

	PBS	UHT milk
D_{65}	33 193	43 732
D_{72}	19 580	21 199
D_{80}	7906	10 591
D_{90}	3127	5577
Z	24.41	27.12

3.2. Reaction order and rate constant

The reaction order, n, and rate constant, k, for denaturation of IGF-I in PBS and UHT milk were determined by examining the closeness of fit of the data for Equation (2) and Equation (3) at different n values. The graphical representation of the heat denaturation process of IGF-I for the reaction order n = 1.1 is shown in Figure 4, and values of k, b and R^2 for n = 1.1 are given in Table II. For n = 1.1, the correlation coefficient, R^2, was ≥ 0.97 and b values were very close to 1. This demonstrated that the alignment of data points was very close to the linear, and the thermal denaturation process of IGF-I followed a reaction order, n = 1.1. Also found in Table II, the value of k increased with heating temperature in both systems, and it was higher in PBS than UHT milk at each temperature.

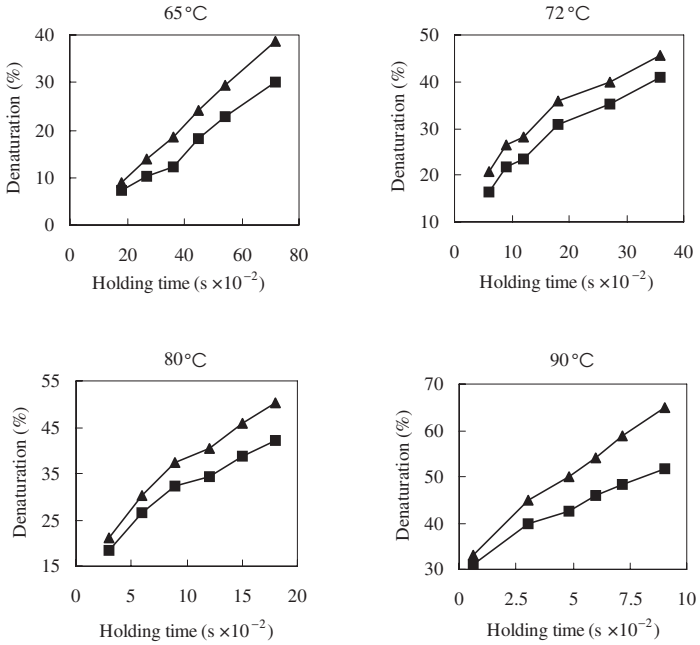

Figure 1. Effect of heat treatment on the denaturation of crude IGF-I in PBS (▲) and UHT milk (■).

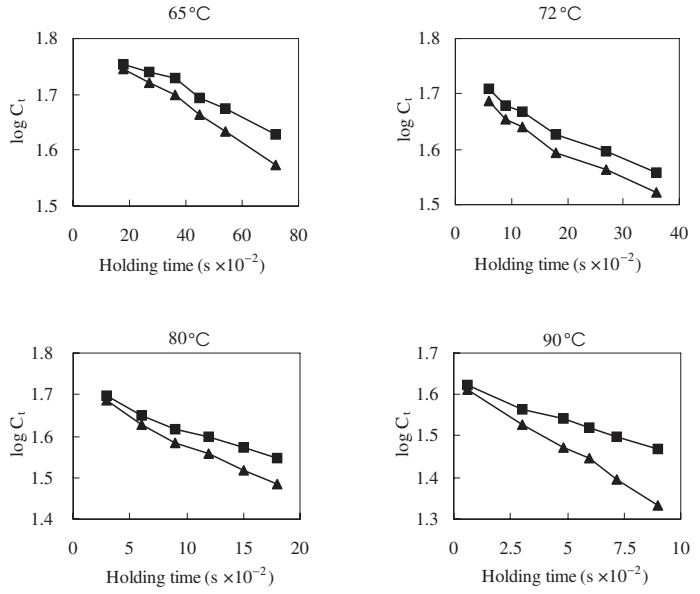

Figure 2. Logarithmic plots of crude IGF-I concentration in PBS (▲) and UHT milk (■) as a function of holding time at 65, 72, 80 and 90 °C.

Table II. Rate constants k (s^{-1}) and b for thermal denaturation of crude IGF-I in PBS and UHT milk and the correlation coefficient R^2 and significance probability *P* value when plotting $(C_t/C_0)^{-0.1}$ versus holding time for the reaction order n = 1.1.

	PBS				UHT milk			
°C	K (× 10^4)	b	R^2	*P*	K (× 10^4)	b	R^2	*P*
65	0.5050	0.9945	0.996	0.0009	0.3672	0.9959	0.981	0.0001
72	0.8292	1.0188	0.974	0.0003	0.7609	1.0136	0.979	0.0002
80	2.0572	1.0169	0.988	0.0001	1.5073	1.0164	0.976	0.0002
90	5.3093	1.0356	0.991	0.0001	2.8900	1.0367	0.990	0.0001

Table III. Kinetic parameters of Ea (kJ·mol^{-1}), ΔH (kJ·mol^{-1}), ΔS (kJ·mol^{-1}·K^{-1}) and ΔG (kJ·mol^{-1}) for thermal denaturation of crude IGF-I in PBS and UHT milk for the reaction order n = 1.1.

	PBS				UHT milk			
°C	Ea	ΔH	ΔS	ΔG	Ea	ΔH	ΔS	ΔG
65	98.02	95.212	–0.03887	108.350	83.86	81.040	–0.08218	108.816
72		95.154	–0.03904	108.622		80.992	–0.08235	109.402
80		95.087	–0.03923	108.935		80.925	–0.08254	110.061
90		95.004	–0.03946	109.329		80.842	–0.08277	110.887

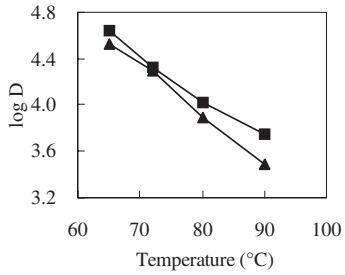

Figure 3. Effect of temperature on D-values for denaturation of crude IGF-I in PBS (▲) and UHT milk (■).

3.3. Kinetic and thermodynamic analysis

The natural logarithmic plots of the rate constant against the inverse of the absolute temperature are shown in Figure 5. A linear relationship was obtained within the temperature ranges. The values of Ea, ΔH, ΔS and ΔG are compared in Table III. The Ea for the thermal denaturation of IGF-I in PBS was higher than in UHT milk (98.02 kJ·mol^{-1} vs. 83.86 kJ·mol^{-1}), which indicates that more low-energy bonds were broken during destruction, and a stronger temperature dependence of IGF-I when heated in PBS than in UHT milk. The higher value for ΔH (95.00 kJ·mol^{-1}) in PBS suggests a larger change in IGF-I conformation during heating in PBS than in UHT milk. In both systems and at all temperatures, the ΔG values were very similar (~108.00 kJ·mol^{-1}). This value was in accordance with the relative constant value of ~100 kJ·mol^{-1} characteristic of the protein denaturation reaction. Also shown in Table III, the ΔS values were about –0.039 kJ·mol^{-1}·k^{-1} and –0.082 kJ·mol^{-1}·k^{-1} in PBS and UHT milk, respectively. The negative ΔS demonstrated that a few intermolecular bonds were formed and the state of order of the system was increased.

4. DISCUSSION

As a functional substance, insulin-like growth factors have a wide range of biological

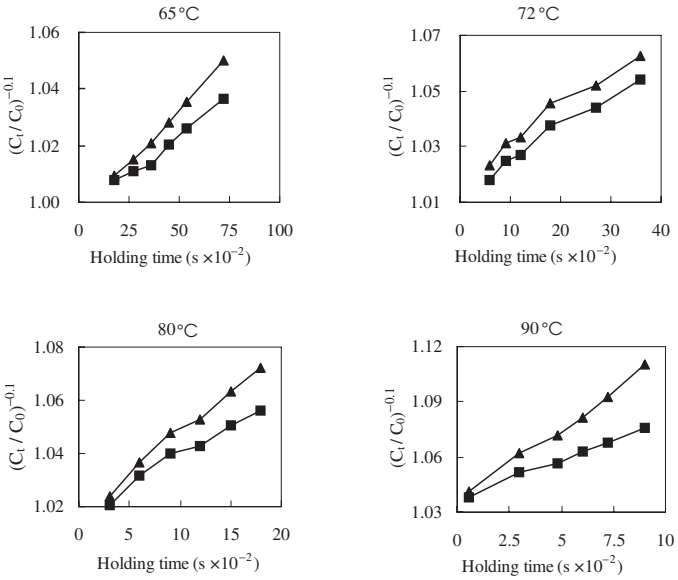

Figure 4. Denaturation of crude IGF-I in PBS (▲) and UHT milk (■) for the reaction order n = 1.1. C_t is the undenatured IGF-I concentration at each holding time and C_0 the initial concentration.

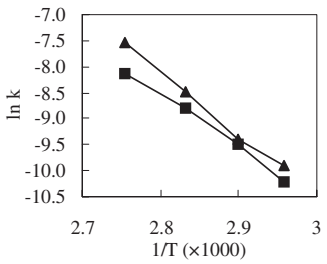

Figure 5. Effect of temperature on rate constant k for denaturation of crude IGF-I in PBS (▲) and UHT milk (■) for the reaction order n = 1.1. T is the absolute temperature.

effects and potential therapeutic values. Several papers have elaborated the clinical use of recombinant human IGF-I (rh IGF-I) [3, 28, 34]. Considering the identical amino acid sequence with rh IGF-I, the biological function and clinical application of bovine milk IGF-I are expected.

The kinetic research for the heat denaturation process enabled prediction of the stability of IGF-I at variant temperatures; hence the selection of the optimal treatment conditions to preserve activity. There is limited research on heat denaturation of IGF-I. Collier et al. [6], Donovan et al. [10] and Elfstrand et al. [11] studied the effect of heating on IGF-I concentration in human milk, bovine milk and bovine colostrums, respectively. However, they only selected one temperature-time combination; namely, 56 °C for 30 min by Collier , 79 °C for 45 s by Donovan and 60 °C for 45 min by Elfstrand, and reported that the IGF-I content was not altered under these specific conditions. Recently, Kang et al. [19] determined the influence of heating on IGF-I concentration in raw bulk milk using a tubular-type heat exchanger. They selected the conditions of heating at 75 °C or 85 °C for 15 min and found that the IGF-I concentration was reduced by 45.0% and 45.2%, respectively. This piece of data was similar to our result (Fig. 1). In this study, we selected four groups of temperature-time combinations, and concluded that bovine milk IGF-I has a high thermal resistance and can survive normal pasteurization conditions in the

dairy industry, such as LTLT (low-temperature, long-time) pasteurization at 63~65 °C for 30 min, and HTST (high-temperature, short-time) treatment at 72 °C for 15 s or 80 °C for 5 s.

The reaction kinetics of thermal denaturation of several whey proteins, such as β-lactoglobulin A and B and α-lactalbumin [1, 7], IgG [16, 22, 23], IgA [23, 24] and lactoferrin [29], have also been reported. Interestingly, the values of Ea and ΔH reported for five whey proteins in a similar temperature range were IgA > IgG > β-lactoglobulin > lactoferrin > α-lactalbumin, with which the order of thermal stability of the proteins were just reversed. In our study, the Ea and ΔH for IGF-I were lower than whey proteins in a similar temperature range. This indicates a higher thermo-resistance of IGF-I than whey proteins in milk. Similarly, the D-values obtained in this study (Tab. I), compared with those reported for whey proteins, confirm the greater thermal resistance of IGF-I than whey proteins.

The thermo-stability of bovine IgG in PBS, boiled milk and UHT milk was investigated by Li-chan et al. [22]. The higher D-values for IgG denaturation in both UHT and boiled milk, compared with PBS, suggested higher stability of IgG in milk than in PBS in the 72 °C to 80 °C range. The thermo-stability of IgG was in good agreement with the results of this study for IGF-I in PBS and milk. However, Sánchez et al. [29] found lactoferrin, with or without bound iron, was more heat-sensitive in milk than in PBS. They attributed the difference partly to a greater change in pH of milk than PBS, observed with increasing temperature. In this study, the change in pH after heating was slight, the changed values being 0.04 units for UHT milk and 0.02 units for PBS. Although the heat-induced pH changes in milk were reversible upon cooling when the heat intensity was not severe, the recovery process was very slow and even needed several hours [36]. Accordingly, the pH value obtained by measuring rapidly cooled UHT milk after heating was approximately the actual pH occurring at the heating temperature, notwithstanding maybe a little higher. Therefore, the effect of pH changes exist, but are negligible.

Milk is a very complex biological fluid containing many complex proteins, lipids, salt, vitamins and carbohydrate systems in soluble, colloidal or emulsified states. These constituents interact with and disturb each other during thermal processing and result in numerous biological, chemical and physico-chemical changes in milk. Kessler and Beyer [20] have shown that the composition of the solution could have a marked effect on the rate constants for the thermal denaturation of β-lactoglobulin and α-lactalbumin. More recently, Despa et al. [8] reported the effect of crowding on the thermal stability of heterogeneous protein solutions through the Lumry-Eyring model theory. They reported that crowding could substantially influence the transition of a protein between its native and unfolded states. In heterogeneous protein solutions containing various types of proteins with different thermal stabilities, the unfolding of the most thermo-labile proteins could enhance the stability of the other proteins in the mixture via the volume exclusion effect. This explanation could be applied to the results of this study. Unfolding of more thermo-labile proteins, such as IgG, bovine serum and β-lactoglobulin, and their cross-linking with k-casein, α_{s2}-casein and α-lactalbumin through a sulfide-disulphide interchange reaction [14], increased the excluded volume of the whole protein system significantly, which could partially contribute to improving the thermal stability of relatively thermo-resistant proteins, such as IGF-I.

It is well known that most of the IGF-I is bound to high-affinity binding proteins (IGFBPs) in milk or in serum. Seven distinct IGFBPs have been identified and each of them has their own special biological roles in different physiological and pathological conditions through IGF-dependent or IGF-independent actions [21]. The exact role of IGFBPs in milk remains to be investigated, but to a certain extent, they can preserve the milk IGF-I from degradation by some proteases [25]. However, up to now, no related research on the effect of IGFBPs on thermal stability of milk IGF-I has been reported. Further experiments are still needed to explore this problem.

Denaturation is commonly used to describe the process of inactivation of native proteins. Denaturation of proteins is defined as a significant change in secondary and tertiary structures. However, sometimes, denaturation often goes along with changes in the primary structure, including the rearrangement and breakage of disulfide bonds, especially occurring at high temperatures [36]. Moreover, electrostatic force, chain elasticity, hydrogen bonding, hydrophobic interactions and sulphydryl-disulphide interchange are all involved in the thermal denaturation process [9]. Therefore, more theoretical and experimental studies will be needed to fully understand the thermal denaturation of proteins.

Acknowledgements: The financial support of the grant No. 30571313 of the National Natural Science Foundation of China for this project is gratefully acknowledged.

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