Journal of Physical and Theoretical Chemistry
of Islamic Azad University of Iran, 7 (1) 27-32: Spring 2010
(J. Phys. Theor. Chem. IAU Iran)
ISSN: 1735-2126

The chaperone ability comparison of norma β-casein and modified β-casein upon interaction with lysozyme

H. Rajabzadeh[1], D. Nourouzian[2], K. Zare[3,*] and F.Mollaamin[4]

[1] Ph.D. Student, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Pasteur Institute of Iran, Karaj Highway, Tehran, Iran
[3] Department of Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran
[4] Department of Chemistry, Qom Branch, Islamic Azad University, Qom, Iran
Received April 2010; Accepted June 2010

ABSTRACT

Diminishing protein aggregation by chaperone is very important factor in medicine and industry. In this paper, it is induced the chaperone ability for β-casein upon modification of its acidic residues by Woodward reagent K(WRK) and examined on lysozyme as a target protein at pH 7.2 and outlined the mechanism for chaperone ability of modified system by UV-Vis and fluorescence spectroscopy and theoretical calculation methods.

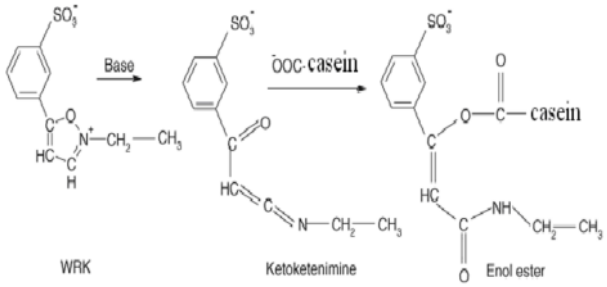
Keywords: Modified β-casein; Lysozyme; Hydrophobicity; Disaggregation; Woodward's reagent K; Accessible surface area
Abbreviations: ANS: 8-Anilino-1-naphthalenesulfonic acid; DTT: Dithiothreiotol; WRK: Woodward's reagent K ASA; Accessible surface area

INTRODUCTION

β-casein and α_s casein have molecular chaperone like properties [1-2]. There are some reports that show caseins decreased turbidity of whey proteins under stress conditions [3]. α_s casein exhibits a considerable anti-aggregation activity [4]. The primary structure of β- casein has a highly amphiphilic character [5]. This is playing a crucial function in aggregation and micellization processes of this casein [6] and β-casein acts also as a surfactant molecule in solution [7]. Because of this property, β-casein is as a natural detergent[7].

There is a short N-terminal hydrophilic polar domain in β-casein chain, that carrying most of the proteins net charges (mostly negative) and a prominent C-terminal hydrophobic domain [8-9].

The approach applied throughout this study is that of the chemical modification of β-casein especially that of carboxyl residues (aspartate and glutamate residues on its surface) which were specifically selected for its effects on diminishing of aggregation for lysozyme. The carboxylic side chain of glutamate and aspartate residues on β-casein surface has been modified using Woodward's reagent K, that is an isoxosolium salt [10-18]. The reaction of WRK with a carboxylate group is outlined in scheme 1. [19].



Scheme 1. Reaction mechanism of WRK.

*Corresponding author: kmzare@gmail.com

The main point to notice is that most recent studies have shown β-casein acts as a molecular chaperone when reacts with the proteins that they have a negative charge on their surface. In fact target-proteins were proteins such as insulin [20, 21] and alcohol dehydrogenase (ADH) [21-23] which have a negative charge on their surface. Since β-casein has a negative charge on its surface, so the electrostatic and hydrophobic factors act in the same direction which caused β-casein molecular chaperone property.

In this work, we attempt to choose lysozyme at pH 7.2 which has 8 positive charges [24] as a target-protein upon the effect of normal and modified β-casein using WRK (aspartate and glutamate residues has been modified) to study the role of electrostatic and hydrophobic forces in chaperone ability of β-casein with lysozyme. We answer to this question that does β-casein play the role of molecular chaperone for the target-proteins with the positive charge on their surface?

MATERIALS

Bovine β-casein, Hen-egg white lysozyme, dithiothreitol (DTT), 2-ethyl-5-phenylisoxazolium-3-sulfonate (Woodward's reagent K), 8-anilino-1-naphthalensulfonic acid (ANS) purchased from Sigma-Aldrich, and other chemicals used were of analytical grade obtained from Merck.

METHODS

Chemical modification of β-casein

Selective modification of aspartic and glutamic acids of β-casein's surface was carried out with WRK. β-casein (0.4 mg/mL) was incubated in 150 mM phosphate buffer (pH 7.2) with concentration of WRK (1 mM) at 37°C and incubated up to 1 h. In order to remove excess WRK, the reaction mixture was dialyzed for 72 h. The above sample treated with WRK had an absorption band with a λ_{max} within 340–350 nm, which was not present in the untreated sample. The stability of WRK is a function of pH, and it rapidly destrays at pH values above 3.0 [25]. For this reason, we used a large excess of reagent over the β-casein in order to compensate the instability of the reagent at pH 7.2 [11].

Chaperone –like activity assay

The aggregation of DTT-induced lysozyme (0.177 mg/ml) in 150 mM sodium phosphate buffer (pH 7.2) was monitored at 360 nm at 37°C for 80 min by Cary Varian UV-Vis spectrophotometer model 100 Bio. In order to reduction of lysozyme aggregation was added 200 µl normal β-casein and modified β-casein (0.4 mg/ml)in to 300 µl reaction solution (lysozyme 0.1% w/w and 10 mM DTT)and carried to 600 µl cuvette.

ANS fluorescence spectroscopy

The ANS fluorescence of lysozyme was recorded on Cary Eclipse Varian fluoresece spectrophotometery. The following solutions were prepared:

a) lysozyme with concentration 0.177 mg/ml in 150 mM phosphate buffer at pH 7.2, lysozyme concentration 0.177 mg/ml in the presence of 0.4 mg/ml modified β-casein, c) lysozyme with concentration 0.177 mg/ml in the presence of 0.4 mg/ml normal β-casein. All above solutions was carried to 0.4 ml cuvette and measured their fluorescence in presence of 10 mM ANS at λ_{ex} 365 nm at 37°C with a 10 nm band width for every measurement.

Theoretical approach

β-casein 3D model generated by the MODELER 9 software based on homology modeling procedure.

To study the modified casein acted on the lysozyme, a WRK attach to an accessible aspartate of casein and then the WRK-casein docked to lysozyme via the HEX5 software. The docking energy would represent estimation of protein-protein interaction affinity. The same procedure performed for unmodified casein.

RESULTS AND DISCUSSION

Molecular chaperones are group proteins and they act not only in protection but also act in folding correction of inappropriately folded, refolding, targeting, and the degradation of defective proteins or their undesired forms [14, 35]. This is due to their binding to the exposed hydrophobic patches of the denaturing / unfolding proteins. It is revealed that bovine milk contains about 80% caseins which 35% is β-casein [27]. It was found that β-

casein has remarkable anti aggregation properties [1, 2], but β-casein hasn't ability to prevent aggregation of lysozyme and doesn't show chaperone ability under our experimental conditions (pH 7.2). In order to revive chaperone ability of β-casein via hydrophobic moiety, aspartate and glutamate residues on its surface were modified using WRK. In fact modification of acidic residues of β-casein induced chaperone properties for modified β-casein upon effect on lysozyme at pH 7.2 in the condition of net negative charges.

The UV-Vis absorbance spectra of the native and WRK modified β-casein are shown in Fig. 1. The modification of the β-casein by WRK shows a new absorption peak in the wavelength range 340-350 nm, establishing the modification of aspartate and glutamate residues [25,28,29]. The first step is formed ketoketenimine,and then reacts with carboxylate group to give enol ester [30]. This indicates an absorbance peak at 340-350 nm at room temperature.

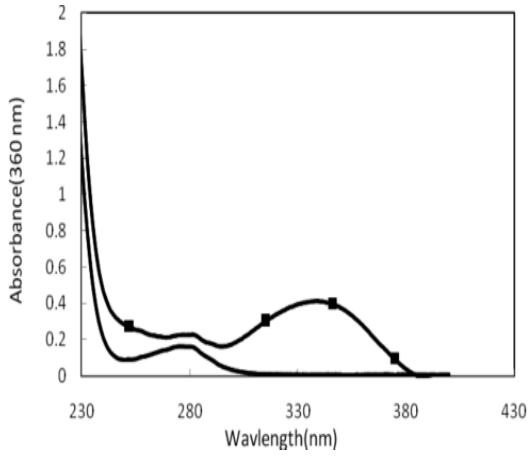


Fig. 1.UV-Vis absorbance spectra of normal and modified β-casein. (—) normal β-casein; (■) β-casein + WRK (1mM); The β-casein concentration was 0.4 mg/ml at pH 7.2 at room temperature.

Fig. 2, shows the status of chemical aggregation of lysozyme in the absence and presence of β-casein, WRK and WRK-modified β-casein at 37°C. However in the presence of modified β-casein aggregation was diminished. Amino acid sequence shows that there are 5 glutamate and 2 aspatate residues on the β-casein surface. The modification of acidic residues does not change the net negative charge due to the existent of WRK sulfonate anion (see scheme 1) [19]. So, what is the reason of aggregation diminishing?

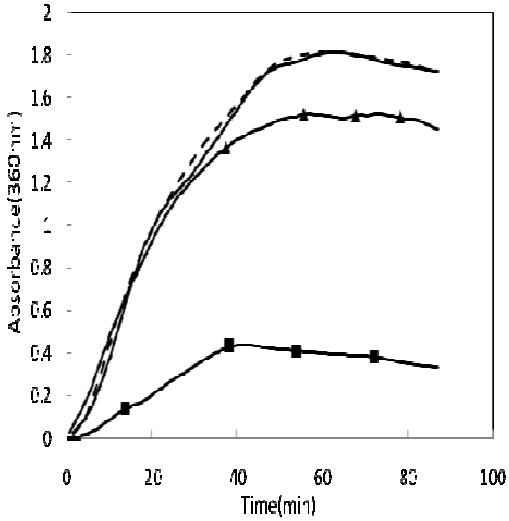


Fig. 2. Chemical aggregation assay of the lysozyme using DTT at the presence of normal and modified β-casein as a function of time at 360 nm at 37◦C . Lysozyme 0.1%(- - - -); lysozyme 0.1% at the resence of normal β-casein 0.4 mg/ml (—); lysozyme 0.1% at the presence of modified β-casein 0.4 mg/ml (■); lysozyme 0.1% at the presence of W RK 30 μM (▲).

In order to illustrate the changes in hydrophobic parts of lysozyme, we used ANS for monitoring the effect of normal β-casein and modified β-casein on hydrophobic parts of lysozyme. Fig. 3, shows that fluorescence intensity which has increased in the presence of modified β-casein and decreased in the presence of normal β-casein. The results obtained from extrinsic fluorescence of lysozyme indicate that there is interaction between exposed hydrophobic patches of modified β-casein and exposed hydrophobic patches of lysozyme, consequently modified β-casein prevents direct interaction between lysozyme hydrophobic patches that caused lysozyme aggregation. Based on ANS fluorescence spectra results, the chemical modification of carboxyl residues caused the conformational change of β-casein. It seems that the hydrophobic and electrostatic interactions play an important role and they compete each other in this case. To understand the mechanism of modified β-casein for diminishing aggregation of lysozyme, we calculated polar ASA and non polar ASA of normal and modified β-casein in lysozyme-β-casein complex. The proteins calculations demonstrate that polar accessible surface area

(ASA) and non-polar ASA of modified β-casein decreases 13% and increases 3% respectively in lysozyme- modified β-casein complex with comparing normal β-casein (see Table 1). Based on image 1, 2 (A) the remained 10% is relative to buried residues at interface between modified β-casein and lysozyme in complex. The image 1 (A) shows modified β-casein electrostatic potential (EP) has not penetrated to the lysozyme core, but normal β-casein electrostatic potential (EP) penetrates to the lysozyme core and fit to it (image 2 A) . The results obtained from calculation (data not shown) reveal that aspartate 152 is more accessible than the other carboxyl residues (glutamate and aspatrate) on β-casein surface. Images 1, 2 (B) show different interaction between aspartate 152 on surface of normal and modified β-casein in β-casein lysozyme complex. In the other words, aspartate 152 on modified β-casein surface is more involving in the interaction with lysozyme, therefore the modified casein has lower electrostatic interaction than the unmodified one. This confirms that the altering of charge position; reduction of ASA polarity and enhancement of hydrophobicity, induced the chaperone ability for modified β-casein upon the interaction with lysozyme at pH 7.2. The change of position and reduction of positive-negative charges interaction revitalized the chaperone ability for β-casein. It has been figured out by our study that, the significance role of charge – charge interaction has been seen in appear or disappear of chaperone activity. This important issue has been pointed at our investigation about molecular chaperone features and launch another factor in studies of chaperone treatment, that charge-charge interaction is an important factor in chaperone ability.

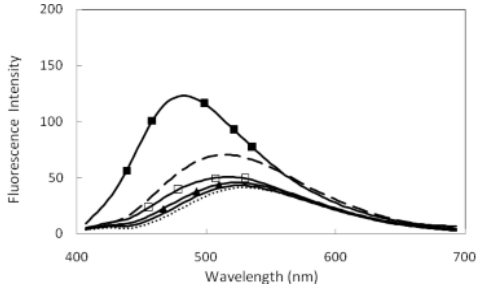


Fig. 3. ANS fluorescence spectra lysozyme 0.1% in the presence of the normal and modified β-casein 0.4 mg/ml in the presence of DTT. Buffer phosphate pH 7.2(….) normal β-Casein (──) modified β-Casein(▲), Lysozyme(─ ─ ─) Lysozyme + normal β-casein (□) lysozyme + modified β-casein (■) at 37◦C.

Table 1. ASA value for normal β-casein, modified β-casein and lysozyme. Delta ASA is defined: difference polar and non polar accessible surface area of normal and modified β-casein in casein-lysozyme complex in percent

	casein	**Lysozyme**	**modified-casein**	**delta ASA (modified casein - casein)**
Polar ASA	4454	2811	3892	-13
non-polar ASA	7582	3752	7827	3
Total ASA	12036	6562	11720	-3

CONCLUSION

It is concluded that positive charge-negative charge interactions remove chaperone ability, while hydrophobic interactions support that. However when the electrostatic interaction is obstacle to appear chaperone activity, by increasing hydrophobic interactions, we can increase chaperone activity. In this study about β-casein, we showed how changing of charge position without changing of net charge, it caused increasing hydrophobic interactions between β-casein and lysozyme and consequently revive chaperone activity of β-casein. Our data clearly indicate that the modified β-casein has chaperone ability and it protects lysozyme against chemical aggregation. It seems that the change of charge position and charge-charge interaction play an important role in preventing aggregation.

A B

Image 1. β-casein-lysozyme-WRK; the dark color SWISS pdb viewer produced electrostatic potential surface indicate modified casein and the light color is lysozyme(A), The tube structures indicate β-casein protein and the ribbon one shows the lysozyme protein. The ball structures used to indicate aspartate 152 and WRK added (B).

A B

Image 2. β-casein-lysozyme; the dark color SWISS pdb viewer produced electrostatic potential surface indicate unmodified casein and the light color one is lysozyme (A), The tube structures indicate β-casein protein and the ribbon one shows the lysozyme protein. The ball structures used to indicate aspartate 152 on unmodified β-casein surface (B).

REFERENCES

[1] Zhang, X.; Fu, X.; Zhang, H.; Liu, C.; Jiao, W.; Chang, Z." Chaperone-like activity of *β*-casein." *Int. J. Biochem. Cell Biol. 37*, 1232–1240, 2005.

[2] Morgan, P. E.; Treweek, T. M.; Lindner, R. A.; Price, W. E.; Carver, J. A." Casein proteins as molecular chaperones." *J. Agric. Food Chem. 53*, 2670–2683, 2005.

[3] O'Kennedy, B. T.; Mounsey, J. S. "Control of heat-induced aggregation of whey proteins using casein." *J. Agric. Food Chem. 54*, 5637–5642, 2006.

[4] Bhattacharyya, J.; Das, K. P. Molecular chaperone-like properties of an unfolded protein, Rs-casein. *J. Biol. Chem. 274*,15505–15509, 1999.

[5] O'Connell, J. E.; Grinberg, V. Y.; de Kruif, C. G. "Association behavior of beta-casein "J Colloid Interface Sci. 258, 33–39, 2003.

[6] Portnaya, I.; Ben-Shoshan, E.; Cogan, U.; Khalfin, R.; Fass, D.; Ramon, D.; Danino, O. D. J Agric Food Chem 56, 2192–2198, 2008.

[7] 3. Gangnard, S.; Zuev, Y.; Gaudin, J.-C.; Fedotov, V.; M.Axelos, A.V.; Chobert, J.-M.; Haertle´, T. Food Hydrocolloids. 21, 180–190, 2007.

[8] Ginger, M. R., & Grigor, M. R. " Comparative aspects of milk caseins." *Comparative Biochemistry and Physiology*, *124*, 133–145, 1999.

[9] Swaisgood, H. E. "Review and update of casein chemistry". *Journal of Dairy Science*, *76*, 3054–3061,1993.

[10] J.L. Arana, R.H. Vallejos."Two different types of essential carboxyl groups in chloroplast coupling factor." FEBS Lett. 123 , 103–106, 1981.

[11] P.H. P´etra, Biochemistry." Modification of carboxyl groups in bovine carboxypeptidase A. I. Inactivation of the enzyme by N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K)" 10, 3163–3170, 1971.

[12] P.H. P´etra, H. Neurath," Modification of carboxyl groups in bovine carboxypeptidase A. II. Chemical identification of a functional glutamic acid residue and other reactive groups" Biochemistry 10, 3171–3177, 1971.

[13] A.J. Brakes, B.H. Weber. " Evidence for an Essential Carboxyl Group for Yeast Phosphoglycerate Kinase reaction with

woodward's reagent K "J. Biol. Chem. 249, 5452–5457, 1974.

[14] K. Llamas, M. Owens, R.L. Blakeley, B. Zerner." N-Ethyl-5-phenylisoxazolium 3'-sulfonate (Woodward's Reagent K) as a reagent for nucleophilic side chains of proteins." J. Am. Chem. Soc. 108, 5543–5548, 1981.

[15] W. Vangrysperre, H. Kersters-Hilderson, M. Callenst, C.K. De Bruyne."Reaction of Woodward's reagent K with D-xylose isomerases Modification of an active site carboxylate residue." Biochem. J. 260, 163–169, 1989.

[16] A.A. Komissarov, D.V. Romanova, V.G. Debabov," Modification with tetranitromethane of an essential tyrosine residue in uridine phosphorylase from *Escherichia coli*. " J. Biol. Chem. 270, 10050–10055, 1995.

[17] P. Paoli, T. Fiaschi, P. Cirri, G. Camci, G. Manao, G. Cappugi, G. Raugei, G. Moneti, G. Ramponi, Biochem. J. 328, 855–861, 1997.

[18] T. Kiss, A. Erdei, L.S. Kiss." Investigation of the Active Site of the Extracellular β-Ī -Xylosidase from *Aspergillus carbonarius*." Arch. Biochem. Biophys. 399, 188–194, 2002.

[19] S. Hashemnia , A.A. Moosavi-Movahedi , H. Ghourchian , F. Ahmad, G.H. Hakimelahi , A.A. Saboury." Diminishing of aggregation for bovine liver catalase through acidic residues modification " International Journal of Biological Macromolecules 40, 47–53, 2006.

[20] Jalil Badraghi, Ali Akbar Moosavi-Movahedi, Ali Akbar Saboury, Reza Yousefi, Ahmad Sharifzadeh, Jun Hong, Thomas Haertlé, Amir Niasari-Naslaji, Nader Sheibani." Dual behavior of sodium dodecyl sulfate as enhancer or suppressor of insulin aggregation and chaperone-like activity of camel αS_1-casein "International Journal of Biological Macromolecules. (2009) in press Mostafa Hassanisadi, Abolfazl Barzegar, Reza Yousefi, Mich`ele Dalgalarrondo, Jean-Marc Chobert, Thomas Haertl´, Ali Akbar Saboury, Ali Akbar Moosavi-Movahedi." Chemometric study of the aggregation of alcohol dehydrogenase and its suppression by β-caseins: A mechanistic perspective" analytica chimica acta 6, 40–47, 2008.

[21] Reza Yousefi,Yulia Y. Shchutskaya, Jaroslaw Zimny, Jean-Charles Gaudin, Ali A. Moosavi-Movahedi, Vladimir I. Muronetz, Yuriy F. Zuev, Jean-Marc Chobert, Thomas Haertle´." Chaperone-like activities of different molecular forms of β-casein. Importance of polarity of N-terminal hydrophilic domain".Biopolymers J.91, 623-632, 2009.

[22] Tomas Koudelka, Peter Hoffmann, John A. Carver. J. Agric. Food Chem. 2009, 57, 5956–5964.

[23] P. Parizadeh, A.A. Moosavi Movahedi, B. Hemmatinejad, F. Ahmad, M. Shamsipur and A.A.Sabouy. "Chemometric study of lysozyme upon interaction with sodium dodecyl sulfate and beta-cyclodextrin" Colloids and Surfaces: Biointerfaces 52, 31-38, 2006 .

[24] U. Sinha, J.M. Brewer," A spectrophotometric method for quantitation of carboxyl group modification or proteins using. Woodward's Reagent K" Anal. Biochem. 151, 327–333, 1985.

[25] A. Carver, R.A. Lindner, C. Lyon, D. Canet, H. Hernandez, C.M. Dobson,C. Redfield," The Interaction of the Molecular Chaperone α-Crystallin with Unfolding α-Lactalbumin: A Structural and Kinetic Spectroscopic Study." J. Mol. Biol. 318, 815–827, 2002.

[26] H.E. Swaisgood, in: P.F. Fox (Ed.)" Chemistry of the Caseins". Advanced Dairy Chemistry-1: Proteins, Elsevier, London, 63–110, 1992.

[27] P. Tomme, M. Claeyssens," Identification of a functionally important carboxyl group in cellobiohydrolase I from *Trichoderma reesei*: A chemical modification study ." FEES Lett. 243, 239–243, 1989.

[28] A.A. Komissarov, D.V. Romanova, V.G. Debabov, J. Biol. Chem. 270, 10050–10055, 1995.

[29] K. Mahnam A.A. Moosavi-Movahedi H. Bahrami G. Hossein Hakimelahi G. Ataie S. Jalili A.A Saboury F. Ahmad S. Safarian M. Amanlou and B. Moshirig" Efficient Factors in Protein Modification: Adenosine Deaminase Esterification by Woodward Reagent K" *J. Iran. Chem. Soc.,* Vol. 5, No. 3, September. 464-475, 2008.