

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Effect of bound ligands and  $\kappa$ -casein  
on the denaturation of  $\beta$ -lactoglobulin**

**A THESIS PRESENTED IN PARTIAL  
FULFILMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY  
IN FOOD TECHNOLOGY AT MASSEY UNIVERSITY**

**YOUNGHEE CHO**

**2000**

## ABSTRACT

The objective of this study was to gain greater understanding of the behaviour of bovine  $\beta$ -lactoglobulin ( $\beta$ -lg) during thermal denaturation. The first part of the study was focused on gaining more knowledge of the structural changes in  $\beta$ -lg during heat-induced and urea-induced denaturation and the effect of ligands and the genetic variants of  $\beta$ -lg. The second part of the study explored the mechanism of the heat-induced interaction of  $\beta$ -lg with  $\kappa$ -casein ( $\kappa$ -CN) and the effects of genetic variants.

The reversible early steps during thermal denaturation of  $\beta$ -lg are not readily separated from the later irreversible steps which involve sulphhydryl-disulphide interchange reactions. However, it should be possible to examine the behaviour of  $\beta$ -lg in the early steps without reactive thiols either by using a porcine  $\beta$ -lg, that does not have cysteine residue equivalent to Cys-121 of bovine  $\beta$ -lg, by blocking the free thiol or by using solvent denaturation.

Heating to 80 °C irreversibly altered near- and far-UV circular dichroism (CD) spectra, and 1,8-anilinonaphthalene sulphonate and retinol binding fluorescence spectra for bovine  $\beta$ -lg, but not for porcine  $\beta$ -lg which unfolded reversibly. Also the examination of the induced CD spectral changes of retinol and *cis*-parinaric acid (PnA) on binding to bovine  $\beta$ -lg B upon heating revealed that bovine  $\beta$ -lg lost the ability to bind retinol and PnA in a chiral environment. In contrast, porcine  $\beta$ -lg did not show significantly induced CD bands upon mixing with retinol, but PnA/ $\beta$ -lg mixtures showed induced CD bands of low intensity. In addition to the lack of a free thiol group in porcine  $\beta$ -lg, the sequence differences between bovine  $\beta$ -lg and porcine  $\beta$ -lg are also likely to affect the behaviour of these  $\beta$ -lgs during heat treatment and the binding of ligands. Although palmitic acid does not show any absorbance in CD spectra, it enhanced the stability of the bovine  $\beta$ -lg and porcine  $\beta$ -lg mixture.

The urea-induced unfolding of bovine  $\beta$ -lg at neutral pH (6.7) revealed that the stability of the genetic variants followed the order,  $\beta$ -lg B <  $\beta$ -lg A <  $\beta$ -lg C, as observed during thermal denaturation and tryptic hydrolysis. The stabilisation effect was also

observed by adding retinol, retinoic acid and palmitic acid during urea denaturation of  $\beta$ -lg, and by retinol, retinyl acetate and PnA during ammonium sulphate denaturation of  $\beta$ -lg. Blocking the sulphhydryl group of  $\beta$ -lg destabilised the native protein against urea denaturation through the introduction of a bulky group to the compact structure of  $\beta$ -lg. This result, together with the results for porcine  $\beta$ -lg, confirms that the sulphhydryl group plays an important role in the unfolding of bovine  $\beta$ -lg.

In the second part of the study, an attempt was made to investigate the effect of bovine  $\kappa$ -CN on the established heat-induced unfolding and aggregation pathway of bovine  $\beta$ -lg, by adding  $\kappa$ -CN A or  $\kappa$ -CN B to native or pre-heated  $\beta$ -lg A, B or C and heating the mixture. The CD band intensity of the mixture of  $\beta$ -lg and  $\kappa$ -CN at 270 nm, an index of significant alteration to the disulphide bond dihedral angle, indicated increasing structural changes involving disulphide bonds during heat treatment.

The rates of loss of  $\beta$ -lg and the distributions of intermediate products were determined using alkaline- and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). During reaction with  $\beta$ -lg, some monomeric  $\kappa$ -CN was found by SDS-PAGE, probably as a result of disulphide bond interchanges between  $\kappa$ -CN and  $\beta$ -lg, and two-dimensional PAGE also revealed disulphide-bonded  $\beta$ -lg/ $\kappa$ -CN aggregates. In the presence of  $\kappa$ -CN, the loss of monomeric  $\beta$ -lg increased and less non-native monomer and dimer were observed compared to  $\beta$ -lg alone and  $\kappa$ -CN reacted more rapidly with  $\beta$ -lg that had been unfolded by prior heat treatment than with native  $\beta$ -lg. This suggested that  $\beta$ -lg probably denatured (unfolded) independently and either simultaneously or consequently and contained higher sulphhydryl reactivity than native  $\beta$ -lg, which lead to the reactions with  $\kappa$ -CN via disulphide bond interchange. It is possible that the equilibrium between native  $\beta$ -lg and denatured  $\beta$ -lg shifted rapidly, because  $\kappa$ -CN preferred to interact with denatured (unfolded)  $\beta$ -lg that has higher sulphhydryl reactivity than native  $\beta$ -lg.

The kinetics of the interaction between  $\beta$ -lg and  $\kappa$ -CN were evaluated from the heat-induced loss of alkaline-monomeric  $\beta$ -lg at 80 °C. The interaction between  $\beta$ -lg and  $\kappa$ -CN could not be described by any reaction order between 1.0 and 2.0. The slopes of the plots changed at about 7.5-10 min heating time and this corresponded to intensity

changes in the alkaline-monomeric, non-native monomeric and dimeric  $\beta$ -lg bands, which increased during the first 10 min of heating and then slightly decreased or remained relatively constant for the rest of the heating.

The loss of native  $\beta$ -lg in  $\beta$ -lg/ $\kappa$ -CN mixtures during heating at 80 °C was shown to be significantly influenced by the genotypes of both  $\beta$ -lg and  $\kappa$ -CN. The  $\kappa$ -CN B variant showed considerably higher reactivity than  $\kappa$ -CN A, while the  $\beta$ -lg B variant was the most reactive. The greatest loss of native  $\beta$ -lg was observed from the  $\beta$ -lg B/ $\kappa$ -CN B mixture.

## ACKNOWLEDGMENTS

I would like to thank my great supervisors, Prof. Harjinder Singh and Dr. Lawrie Creamer for their guidance and assistance in all aspects of this course. I have learned not only this lovely subject ( $\beta$ -lactoglobulin) but also the attitude of real scientist from them. Thanks for always there for me and your encouragement during last three years. I never could thank enough to you.

I am thankful to Dr. Gavin Manderson who helped me to set up and run my experiment during my first few weeks at NZDRI. Thanks to Dr. John Lucey for teaching me about SEC-MALLS. I also thank Dr. Don Otter for helping me to run mass spectrometry, Dr. Paul Harris for introducing me isoelectric focusing technique and Dr. Skelte Anema for giving me useful tips about SigmaPlot<sup>to the</sup>. Grateful thanks go to Dr. Palatasa Havea for sharing knowledge of 2D-PAGE as well as his friendship and valuable advice throughout this course. I also thank the members of the Food Science Section (NZDRI) for their help and friendship during course of this project (Marlene, Michelle, Robyn, Carmen, Michael, Christina and Nicola). Thanks guys!

I thank for the financial support by the Foundation of Research, Science and Technology (Contract number DRI 403). I also like to thank Dr. Jeremy Hill (Section manager, Food Science Section, NZDRI) for the opportunity to conduct research at NZDRI.

I would like to express my genuine gratitude to my parents and father-in-law in Seoul for their support and love. Finally, I have to thank to my lovely and wonderful husband, Dr. Sang-dong Yoo, for his love and patience.

## CONTENTS

Abstract	i
Acknowledgements	iv
Contents	v
<b>Chapter 1. Introduction</b>	<b>1</b>
<b>Chapter 2. Literature review</b>	<b>3</b>
2.1. Bovine $\beta$ -lactoglobulin	3
2.2. Structure of $\beta$ -lactoglobulin	4
2.2.1. Primary structure and genetic variants of $\beta$ -lactoglobulin	4
2.2.2. Secondary and tertiary structures of $\beta$ -lactoglobulin	6
2.2.3. Binding properties of $\beta$ -lactoglobulin	9
2.3. Conformational changes of $\beta$ -lactoglobulin in solution	13
2.3.1. Self-association of $\beta$ -lactoglobulin	13
2.3.2. pH-induced reversible conformational changes of $\beta$ -lactoglobulin	14
2.3.3. Reversible unfolding of $\beta$ -lactoglobulin	15
2.3.4. Thermal unfolding and aggregation of $\beta$ -lactoglobulin	15
2.3.4.1. Thermal unfolding of $\beta$ -lactoglobulin	15
2.3.4.2. Thermal aggregation of $\beta$ -lactoglobulin	19
2.3.4.3. Comparison of thermal susceptibilities of $\beta$ -lactoglobulin variants	23
2.4. Effect of heat treatment on mixtures of whey proteins	24
2.5. Heat-induced interactions between $\beta$ -lactoglobulin and $\kappa$ -casein	25
2.5.1. Effect of heat on individual caseins	25
2.5.2. Characterisation of $\kappa$ -casein	26
2.5.2.1. Structure of $\kappa$ -casein	26
2.5.2.2. Genetic variants of $\kappa$ -casein	28
2.5.3. Interactions of casein with whey proteins	30
2.5.4. $\beta$ -Lactoglobulin and $\kappa$ -casein complex formation	32
2.5.5. Effect of genetic variants on the interaction between $\beta$ -lactoglobulin and $\kappa$ -casein	34
2.6. Research objectives	35
<b>Chapter 3. Materials and methods</b>	<b>37</b>
3.1. Materials	37
3.2. General methods	38
3.2.1. Electrophoresis	38

3.2.1.1. SDS-PAGE	38
3.2.1.2. Alkaline-PAGE	41
3.2.1.3. Two-dimensional PAGE	42
3.2.1.4. Alkaline-urea PAGE	43
3.2.1.5. Borate PAGE	44
3.2.1.6. Isoelectric focusing	46
3.2.2. Circular dichroism spectroscopy	48
3.2.3. Fluorescence spectroscopy	48
3.2.4. Mass spectrometry	49
<b>Chapter 4. Development of isolation and purification methods for</b>	
<b><math>\beta</math>-lactoglobulin and <math>\kappa</math>-casein</b>	<b>51</b>
4.1. Preparation of $\beta$ -lactoglobulin	51
4.1.1. Introduction	51
4.1.2. Materials and methods	53
4.1.2.1. Materials	53
4.1.2.2. $\beta$ -Lactoglobulin purification methods	53
4.1.2.3. General methods	56
4.1.3. Results and discussion	58
<i>I. Phenotyping and purification of crude <math>\beta</math>-lactoglobulin</i>	58
4.1.3.1. Phenotyping of $\beta$ -lactoglobulin	60
4.1.3.2. Size-exclusion chromatography	60
4.1.3.3. Yields of $\beta$ -lactoglobulin	63
<i>II. Characterisation of purified <math>\beta</math>-lactoglobulin</i>	63
4.1.3.4. SEC-MALLS	63
4.1.3.5. Mass spectrometry	65
4.1.3.6. Near-UV CD	69
4.1.3.7. Far-UV CD	73
4.1.3.8. ANS fluorescence	76
4.1.4. Conclusions	78
4.2. Preparation of $\kappa$ -casein	79
4.2.1. Introduction	79
4.2.2. Materials and methods	80
4.2.2.1. Materials	80
4.2.2.2. Preparation of $\kappa$ -casein	80
4.2.3. Results and discussion	82
4.2.3.1. Alkaline-urea PAGE	82
4.2.3.2. Size-exclusion chromatography	84
4.2.3.3. Addition of soybean trypsin inhibitor	84



## **PART I. ROLE OF LIGANDS AND FREE CYSTEINE IN $\beta$ -LACTOGLOBULIN DENATURATION**

<b>Chapter 5. Effect of heat treatment on the structure and ligand-binding properties of bovine <math>\beta</math>-lactoglobulin and porcine <math>\beta</math>-lactoglobulin</b>	<b>87</b>
5.1. Introduction	87
5.2. Experimental protocol	88
5.2.1. Preparation of porcine $\beta$ -lactoglobulin	88
5.2.2. Measurement protocols	90
5.3. Results and discussion	92
5.3.1. Structural changes in bovine and porcine $\beta$ -lactoglobulin during heating and subsequent cooling	92
5.3.1.1. Unheated bovine and porcine $\beta$ -lactoglobulin	92
5.3.1.2. Heat-treated bovine and porcine $\beta$ -lactoglobulin	95
5.3.2. Binding properties of bovine and porcine $\beta$ -lactoglobulin during heating and subsequent cooling	98
5.3.2.1. Near-UV CD	98
(i) Retinol	98
(ii) cis-Parinaric acid	103
(iii) Palmitic acid	108
5.3.2.2. Ligand fluorescence	110
(i) ANS fluorescence	112
(ii) Retinol fluorescence	117
5.3.3. General discussion	119
<b>Chapter 6. Effect of urea concentration on the unfolding of different genetic variants of <math>\beta</math>-lactoglobulin and ligand-bound <math>\beta</math>-lactoglobulin</b>	<b>125</b>
6.1. Introduction	125
6.2. Experimental protocol	126
6.3. Results and discussion	128
6.3.1. Effect of urea concentration on the unfolding of $\beta$ -lactoglobulin	128
6.3.1.1. Near-UV CD	128
6.3.1.2. Far-UV CD	134
6.3.2. The role of the thiol group in the urea-induced unfolding of $\beta$ -lactoglobulin	137
6.3.2.1. Thiol-blocked $\beta$ -lactoglobulin	138
(i) $\beta$ -Lactoglobulin blocked by DTNB	138
(ii) $\beta$ -Lactoglobulin blocked by NEM	141
6.3.2.2. Effect of urea concentration on the unfolding of thiol-blocked $\beta$ -lactoglobulin	146

6.3.3. Effect of urea concentration on the unfolding of ligand-bound $\beta$ -lactoglobulin	154
6.3.3.1. Near-UV CD	154
(i) Retinol	154
(ii) Retinoic acid	154
(iii) Palmitic acid	157
6.3.3.2. Far-UV CD	158
(i) Retinol	158
(ii) Retinoic acid	158
(iii) Palmitic acid	160
6.3.4. General discussion	160

## **Chapter 7. Conclusions – Part I** **164**

## ***PART II. HEAT-INDUCED INTERACTIONS BETWEEN $\beta$ -LACTOGLOBULIN AND $\kappa$ -CASEIN***

<b>Chapter 8. Heat-induced interactions of <math>\beta</math>-lactoglobulin A and <math>\kappa</math>-casein B</b>	<b>168</b>
8.1. Introduction	168
8.2. Experimental protocol	170
8.3. Results and discussion	171
8.3.1. Structural changes during heating and cooling of $\beta$ -lactoglobulin A and $\kappa$ -casein B	171
8.3.2. Effect of heat treatment on $\beta$ -lactoglobulin A and $\kappa$ -casein B	175
8.3.2.1. Effect of heat treatment on $\beta$ -lactoglobulin A	175
8.3.2.2. Effect of heat treatment on $\kappa$ -casein B	177
8.3.3. Effect of heat treatment on $\beta$ -lactoglobulin A and $\kappa$ -casein B mixtures	179
8.3.3.1. Alkaline-PAGE	179
8.3.3.2. SDS-PAGE	181
8.3.3.3. 2D-PAGE	183
8.3.3.4. Quantitative analysis	185
8.3.4. Heat-induced interactions between pre-heated $\beta$ -lactoglobulin A and native $\kappa$ -casein B	188
8.3.4.1. Alkaline-PAGE	188
8.3.4.2. SDS-PAGE	190
8.3.4.3. High molecular weight SDS-PAGE	192
8.3.5. General discussion	192

<b>Chapter 9. Effect of genetic variants on the heat-induced interactions between <math>\beta</math>-lactoglobulin and <math>\kappa</math>-casein</b>	<b>196</b>
9.1. Introduction	196
9.2. Experimental protocol	197
9.3. Results and discussion	197
9.3.1. Effect of genetic variants and $\beta$ -lactoglobulin: $\kappa$ -casein ratios on the heat-induced interaction between $\beta$ -lactoglobulin and $\kappa$ -casein	197
9.3.1.1. Heat treatment of $\beta$ -lactoglobulin variant solutions at various concentrations	199
9.3.1.2. Heat treatment of $\beta$ -lactoglobulin and $\kappa$ -casein variant mixtures at various concentrations	213
9.3.2. 2D-PAGE	216
9.3.3. Reaction kinetics of heat-induced interactions between $\beta$ -lactoglobulin and $\kappa$ -casein	219
9.3.4. Heat-induced interactions between pre-heated $\beta$ -lactoglobulin and native $\kappa$ -casein	224
9.3.5. General discussion	230
<b>Chapter 10. Conclusions – Part II</b>	<b>233</b>
<b>References</b>	<b>236</b>
<i>Appendix 1.</i> The effect of ammonium sulphate concentration on the unfolding of ligand-bound $\beta$ -lactoglobulin	257
A1.1. Introduction	257
A1.2. Experimental protocol	257
A1.3. Results and discussion	258
A1.3.1. Retinol	258
A1.3.2. Retinyl acetate	260
A1.3.3. cis-Parinaric acid	262
<i>Appendix 2.</i> Kinetic evaluation of the heat-induced interactions between $\beta$ -lactoglobulin and $\kappa$ -casein at various concentrations	264
A2.1. Introduction	264
A2.2. Experimental protocol	264
A2.3. Results	264