# Non natural fatty acids binding affinity to bovine β-lactoglobulin: Crystallographic and thermodynamic studies

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## INTRODUCTION

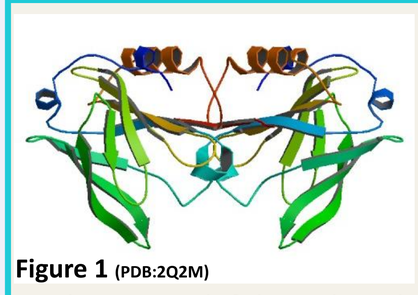

**Figure 1** (PDB:2Q2M)

Bovine β-lactoglobulin (β-Lg) (Fig.1) is a globular protein of 18.4 kDa, consisting of 162 amino acids. It is the major whey protein in bovine milk but it is absent in human milk. Structurally, β-Lg belongs to the lipocalin family, in which all members share a characteristic up-and-down eight-stranded β-barrel often called as calyx.

The central β-barrel makes the hydrophobic scaffold of the lipocalin molecule and is the primary binding pocket for hydrophobic ligands, such as fatty acids, and hydrophobic vitamins (Fig.2) [1]. Fatty acids are the most abundant endogenous ligands of β-Lg [2].

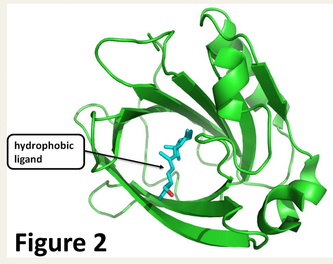


**Figure 2**

In the past decade, there has been a significant body of work on this protein, further defining its structure, properties and increasingly, its applications in the general area of food and nutrition [3,5]. Despite intensive studies on biological, chemical, and physical properties of this protein, its biological function still remains unknown [4].

The aim of the present study was the examination of the binding affinity of β-Lg to non-natural fatty acids. Thermodynamic and structural studies of β-Lg complexes with: tri-, penta-, hepta- and nona-decanoic acid (C13, C15, C17 & C19, respectively) were conducted. Crystal structures of β-Lg complexes with these non natural fatty acids were obtained. The interactions of the above mentioned fatty acids with β-Lg were also studied by isothermal titration calorimetry and $K_d$ values were determined.

## MATERIALS & METHODS

**Crystallization:** Crystals of β-Lg complexes with tridecanoic (β-Lg–C13) were obtained in hanging drop setup. 4 μL of β-Lg solution (20mg/mL) + C13 (10mM) in 20mM Tris-HCl buffer, pH 8 were mixed with 4 μL of well solution. Drops were equilibrated against 1000 μL of well solution containing 1M Sodium Citrate & 0.1M Hepes, pH 7.5.

**X-ray data collection:** Data were collected at 10 °C (120 K) on an X-ray generator Oxford Diffraction, equipped with microfocus Cu Kα (1.54 Å) X-ray source working at 50 kV and 1 mA and 135 mm CCD detector.

**Structure solution and refinement:** The refinement calculation of crystal structures of β-Lg–C13 complexes was interleaved with rounds of model-building with the programs REFMAC & COOT [6,7]. Water molecules were added using the program COOT [7].

**Isothermal Titration Calorimetry (ITC):** The interactions of fatty acids with β-Lg were studied by ITC at 25 °C (298 K) using the Nano ITC instrument (TA Instruments, USA). Stock solutions of the fatty acids were prepared in ethanol and diluted before measurement with 50mM Tris–HCl buffer (pH 8) to the concentration below their critical micelle concentration (CMC). The protein solutions were supplemented with ethanol. The sample cell was loaded with the solution of each fatty acid and the β-Lg protein was placed in the injection syringe. Data analysis was performed using NanoAnalyze scientific plotting software according to model of the single set of identical independent sites. Standard deviations of $K_a$, $K_d$, n, ΔH & ΔS were calculated from three titration runs for all fatty acids (Table 1).

## RESULTS

**Overall crystal structure:**

Crystals of β-Lg complexes with C13 grew in the above mentioned conditions (Fig.3). Crystals of β-Lg complexes with C15, C17 and C19 were also obtained (data not shown).

- The β-Lg–C13 structures determined to resolution 2.10 Å and the first difference electron density map ($2F_o - 1F_c$) revealed that the central calyx binding site was occupied by a single fatty acid molecule (Fig. 4).
- The structure show that end of ligand hydrocarbon chain is always bound deep in the hydrophobic part of β-barrel while carboxyl group is located near the calyx entrance.
- The central calyx is the only observed binding site in structure of β-Lg complexes with the studied fatty acid. Binding of C13 to β-Lg did not affect significantly conformation of protein main chain.

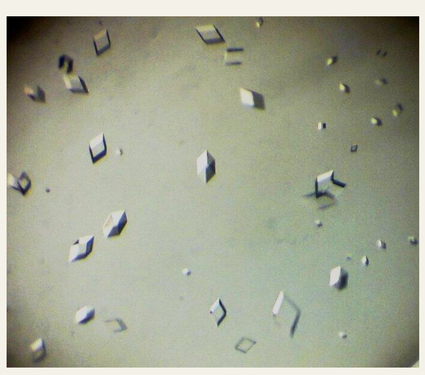

**Figure 3**

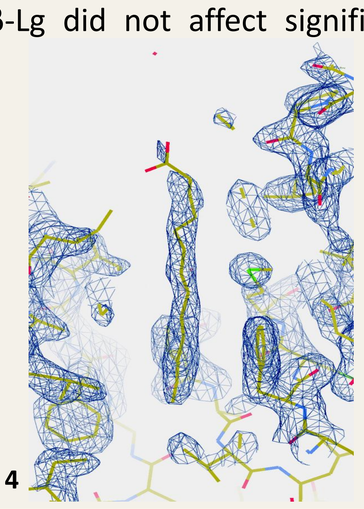

**Figure 4**

**Thermodynamics of C13, C15, C17 & C19 binding to β-Lg:**

The enthalpic component depends mostly on interactions between fatty acid, solvent and protein while the change of entropy is related to effects associated with conformational changes of ligand, solvent displacing from the binding site or solvent relief when fatty acid enters the binding cavity.

- The determined association constants are similar for all complexes (Table 1).
- The values of thermodynamic parameters indicated that:
  - binding of C13 & C15 to β-Lg was both enthalpically and entropically driven
  - binding of C17 and C19 to β-Lg was primarily enthalpically driven.
- The ITC results for the four studied fatty acids showed that their binding to β-Lg is spontaneous and exothermic (Table 1).
- Experimental data were fitted to one-site binding model (Fig. 5).

The different observed saturation peak heights of each fatty acids are due to different ethanol % concentration solutions used for each fatty acid (due to different fatty acids aqueous solubility).

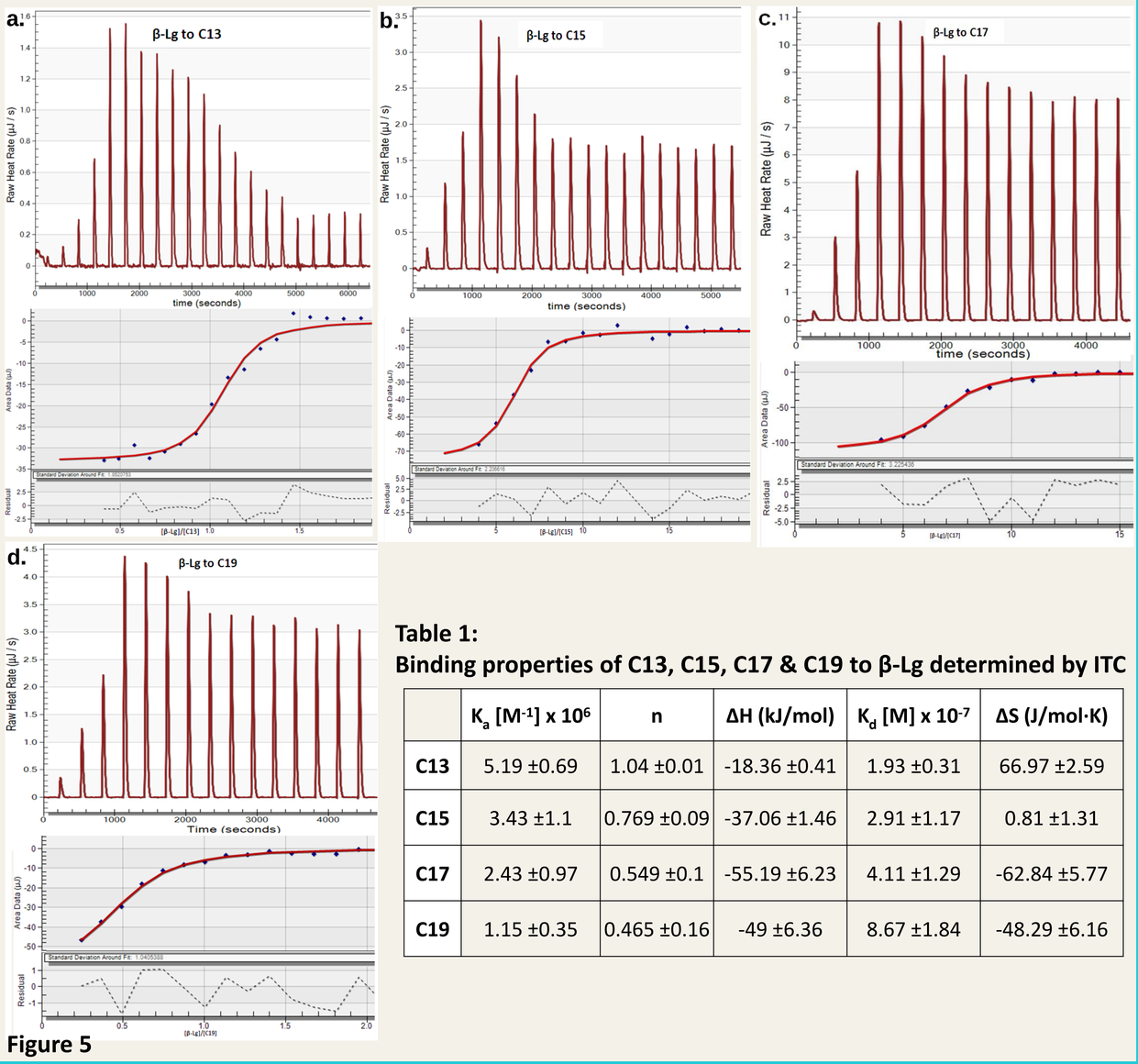


**Figure 5**

**Table 1:**
**Binding properties of C13, C15, C17 & C19 to β-Lg determined by ITC**

| | $K_a$ [$M^{-1}$] x $10^6$ | n | ΔH (kJ/mol) | $K_d$ [M] x $10^{-7}$ | ΔS (J/mol·K) |
|---|---|---|---|---|---|
| **C13** | 5.19 ±0.69 | 1.04 ±0.01 | -18.36 ±0.41 | 1.93 ±0.31 | 66.97 ±2.59 |
| **C15** | 3.43 ±1.1 | 0.769 ±0.09 | -37.06 ±1.46 | 2.91 ±1.17 | 0.81 ±1.31 |
| **C17** | 2.43 ±0.97 | 0.549 ±0.1 | -55.19 ±6.23 | 4.11 ±1.29 | -62.84 ±5.77 |
| **C19** | 1.15 ±0.35 | 0.465 ±0.16 | -49 ±6.36 | 8.67 ±1.84 | -48.29 ±6.16 |

## CONCLUSIONS

- Both crystallographic and thermographic studies indicate that β-Lg formed complexes with the four studied non natural fatty acids.
- β-Lg's ability to bind non-natural hydrophobic compounds (except of the already known wide spectrum of natural ligands), facilitate its functionality as:
  1. a protein that solubilizes hydrophobic molecules and
  2. makes it a promising candidate as:
     - a protector for sensitive hydrophobic nutraceuticals in food products during processing;
     - a carrier in advancing nanotechnology for food and drug applications (e.g., oral delivery of cancer therapy)

## REFERENCES

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