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# Effect of high hydrostatic pressure on the steady-state kinetics of tryptic hydrolysis of β-lactoglobulin

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**Abstract**

The rate of hydrolysis of β-lactoglobulin (genetic variant B) by trypsin in aqueous solution at pH of 7.6 and ionic strength 0.08 M (NaC1) at 25.0 °C increased with increasing hydrostatic pressure. At constant pressure (ambient, 100, 150 and 250 MPa studied), the initial rate of disappearance of β-lactoglobulin B could be analyzed using Michaelis–Menten kinetics (five initial substrate concentrations ranging from 0.11 to 0.88 mM used at each pressure). The over-all catalytic efficiency (ratio between catalytic constant $k_c$ and Michaelis constant $K_m$) increased by a factor of $10^3$ at 250 MPa compared with ambient pressure. While $k_c$ increased only for pressures up to 100 MPa, corresponding to an initial pressure-melted state (factor of $10^2$ up to 100 MPa) with no further increase at higher pressure, $K_m$ continuously decreased up to 250 MPa, corresponding to a binding volume of $-37$ ml mol$^{-1}$ of β-lactoglobulin to trypsin. Thus, the enhanced digestibility of β-lactoglobulin at elevated pressure is the result of a volume decrease during the association process and a volume decrease during the catalytic step. Pressure treatment of whey concentrates should be considered as an alternative processing technology for reduction of allergenicity of whey products.


*Keywords:* β-Lactoglobulin B; Trypsin; steady-state kinetics; High hydrostatic pressure

## 1. Introduction

During the past decade, high pressure treatment of foods and food components has gained increasing interest as a non-thermal method of modifying the structure and functional properties of food macromolecules, such as proteins, without affecting the flavour, colour and content of vitamins. Modification of proteins by high hydrostatic pressure depends on dissociation and denaturation of oligomeric proteins by increased water-binding of the individual subunits, in effect causing a decrease in the volume of the protein due to increased hydration (Weber, 1987; Wong, Saint Giron, Guillou, Cohen, Barzu, & Mantsch, 1989).

The susceptibility of a protein to denaturation is dependent on its structure (Marshall, 1982; McSwiney, Singh, Campanella, & Creamer, 1994), and the very different responses to hydrostatic pressure observed for various food proteins, have been rationalized on the basis of differences in the compactness of the pressure-perturbed structure (Tanaka, Koyasu, Kobayashi, & Kunugi, 1996). Among the milk proteins, β-lactoglobulin appears to be most sensitive to hydrostatic pressure, and has, by in situ study of fluorescence depolarization at high pressure, been found to be half denatured at the moderately high pressure of 123 MPa at neutral pH (Stapelfeldt & Skibsted, 1999). As the major whey protein, β-lactoglobulin is an important byproduct from cheese production. However, β-lactoglobulin has been recognised as an allergenic component of bovine milk and, moreover, it is rather resistant to hydrolysis by key digestive proteases. It is accordingly of interest to study the interaction between β-lactoglobulin and proteases at higher pressure, in order to develop processes where β-lactoglobulin can be partly hydrolyzed with the objective of decreasing its allergenicity.

It has been reported, that β-lactoglobulin in whey concentrates is preferentially hydrolyzed by thermolysin under high pressure compared with the other whey proteins (Hayashi, Kawamura, & Kunugi, 1987). Hydrolysis of β-lactoglobulin by thermolysin or pepsin

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at pressures in the range 0.1–350 MPa has been found to show a significant increase in rate with increasing pressure. However, it was observed that the proteolytic actions of thermolysin and pepsin during pressurization were different, as pressure both affects the enzyme and partly unfolds the substrate (Dufour, Hervé, & Haertle, 1995). Thus, detailed studies are necessary in order to resolve the complex enzyme kinetics of pressure-induced hydrolysis of β-lactoglobulin. Some studies of the susceptibility of β-lactoglobulin to enzymatic hydrolysis at high pressure have reported apparent volumes of activation for the hydrolysis (Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996; Van Willige & Fitzgerald, 1995). However, no steady-state kinetic experiments have been performed with the goal of investigating the influence of pressure on enzymatic digestion of β-lactoglobulin. Thus no attempts have been made to assign effects of pressure to individual steps in the enzymatic process, although it has been demonstrated that the increase in hydrolysis of β-lactoglobulin by trypsin, thermolysin or pepsin is not due to enzyme activation, but rather due to effects on the substrate and the substrate/enzyme interaction (Stapelfeldt et al., 1996).

In the present study, the initial proteolysis of a highly pure preparation of β-lactoglobulin B at ambient and high pressures was investigated. The steady-state kinetics of the tryptic hydrolysis of β-lactoglobulin was analysed according to the Michaelis–Menten equation, and the effects of pressure on the kinetic parameters were resolved, in order to determine whether the increased digestibility of β-lactoglobulin at increased pressure is mainly due to effects of pressure on β-lactoglobulin, on the enzyme, or on the enzyme–substrate interaction.

## 2. Materials and methods

### 2.1. Chemicals

β-Lactoglobulin from bovine milk, genetic variant B, was isolated from acidic whey of fresh skim milk of homozygotic cows, from the field station of the Royal Veterinary and Agricultural University in Taastrup, and purified according to the method described by Kristiansen, Otte, Ipsen, and Qvist (1998). In order to avoid denaturation, the temperature of any purification step never exceeded 45 °C, and the final product was lyophilized. Analytical grade tris[hydroxymethyl]aminomethane (Tris) and tosyl phenylalanine chioromethyl ketone treated trypsin (TPCK-trypsin, 10,000 BAEE U/mg) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Analytical grade dithiothreitol (DTT) and urea were obtained from Merck (Darmstadt, Germany). Soybean trypsin inhibitor was from Boehringer Mannheim (D-68298 Mannheim, Germany). All other chemicals were of analytical grade, and solutions were made with highly purified water (Milli-Q Plus, Millipore Corp, Bedford, MA, USA).

### 2.2. Pressure treatment and hydrolysis

Trypsin-catalyzed hydrolysis of β-lactoglobulin B was studied at 25.0 °C in a buffer consisting of 50 mM Tris, pH 7.8 and ionic strength 0.08 M (NaC1). The rates of hydrolysis were measured in triplicate at five concentrations of β-lactoglobulin B (0.11, 0.22, 0.44, 0.66, and 0.88 mM) using enzyme concentrations of 51 nM or 4.0 μM at high pressure or ambient pressure, respectively. In the hydrolysis experiments performed at high pressure, 200 μl of a solution of trypsin (0.56 μM in 50 mM Tris, pH 7.8) were added to 2.0 ml of solutions with each of the concentrations of β-lactoglobulin B and the reaction mixtures were incubated at 25.0 °C at atmospheric pressure for 5 min. To a reference sample of each concentration of β-lactoglobulin was added 200 μl of buffer. The reaction mixtures were immediately transferred to flexible polyethylene tubes (1.5 ml, Nalgene, Nalge Nunc International, USA), completely filled and closed with a screw cap equipped with an O-ring. Samples were pressurized at 25.0 °C in a thermostatted pressure reactor (PSIKA Systems Ltd., Stanford, UK) by using water as a pressuring medium at pressures of 100, 150, or 250 MPa. In all experiments, the rate of pressure increase was approximately 100 MPa/min in order to minimize adiabatic heating. After 0, 5, 10, 15, 20, 25 or 30 min of hydrolysis the pressure was quickly released and aliquots (0.20 ml) of the hydrolysates were immediately added to 25 μl of trypsin-inhibitor (8.5 μM in Tris-buffer) to quench the enzyme activity.

The hydrolysis experiments performed at ambient pressure, were carried out essentially as described by Olsen, Otte, and Skibsted (2000). A trypsin solution (0.17 mM in Tris-buffer; 50 μl) was added to 2.00 ml of each substrate concentration of β-lactoglobulin B and aliquots (0.15 ml) were withdrawn after various times of hydrolysis and the reaction was quenched as described earlier. All samples from hydrolysis experiments were stored at −20 °C until subsequently analyzed.

### 2.3. Capillary electrophoresis

Hydrolysis of β-lactoglobulin was quantified by capillary zone electrophoresis using a Waters Quanta 4000 CE instrument (Waters, Milford, MA, USA), equipped with an untreated 50 μm i.d. fused silica capillary, 60 cm long, with 52.5 cm to the detector window. A 10 mM sodium phosphate buffer with 6.0 M urea, pH 2.7, was used and the analysis was performed at room temperature. Samples were injected hydrodynamically at the anode (20 s, by rising the end of the capillary 10 cm in order to obtain 10 mbar). Samples

were pretreated by one of two procedures in order to dissolve pressure-induced aggregates of β-lactoglobulin B, produced as a result of sulphydryl/disulfide exchange, and in order to ensure that all β-lactoglobulin B was in its monomeric form: (i) β-lactoglobulin B sample was mixed (1:1) with 8.0 M urea and 20 mM DTT in the same buffer as used for the sample; or (ii) Samples were mixed (1:4) with 10 M urea and 25 mM DTT in water and left at room temperature for reaction at least 1 h before analysis. Quantification was based on standard curves made with varying concentrations (2–16 mg/ml) of pure β-lactoglobulin B in the sample buffer.

### 2.4. Kinetic analysis

The initial rate of hydrolysis of β-lactoglobulin B was calculated from the disappearance during proteolysis by trypsin, taking the integrated peak area of the electropherograms as the relative concentration of β-lactoglobulin B. On the basis of standard curves of pure β-lactoglobulin B in sample buffer, the peak areas were transformed to concentrations and the rate of hydrolysis was described by fitting a second order polynomial to the data. The initial rate of hydrolysis was obtained as the first derivative of the polynomial with respect to time of start of the hydrolysis. The steady-state kinetic parameters, $k_c$ and $K_m$, were calculated from fits of the Michaelis–Menten equation to the initial rate of hydrolysis as a function of substrate concentration, using the non-linear least-squares fitting program GraFit (Erithacus Software Ltd., UK).

## 3. Results and discussion

The gradual disappearance of β-lactoglobulin B, hydrolyzed by trypsin, was followed by CE as a function of time. The electropherograms obtained during the hydrolysis of β-lactoglobulin B at 250 MPa are shown as an example in Fig. 1, in which unhydrolyzed β-lactoglobulin B corresponds to the peak with migration time around 21 min.

The rate of hydrolysis of β-lactoglobulin decreased with time, as is normally observed for enzymatic processes (Fig. 2). The decrease in concentration of the substrate, β-lactoglobulin B, and possible competitive inhibition of the enzyme by the products formed during proteolysis, together account for this effect. For this reason, the kinetic data were calculated using the initial rates of hydrolysis as determined by differentiation, at time zero, of a polynomial interpolation of concentration as a function of time. Accordingly, the steady-state conditions prevailing at the initiation of the reaction, where the concentration of β-lactoglobulin B can be considered as constant and the concentration of

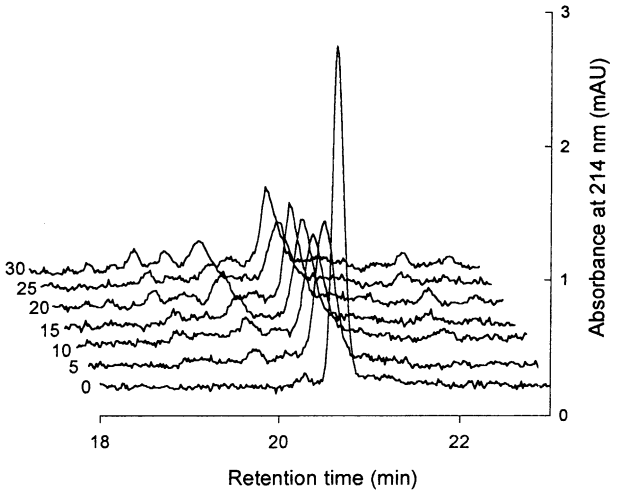


Fig. 1. Separation by capillary electrophoresis of the tryptic hydrolysate of β-lactoglobulin B obtained after different times of hydrolysis, in min at 250 MPa. Intact β-lactoglobulin is seen as the peak area with migration time around 21 min. Reaction conditions were 50 mM Tris, pH 7.6, ionic strength 0.08 M (NaCl), at 25.0 °C with a substrate concentration of 2.0 mg/ml and 51 nM trypsin. For details, see Section 2.

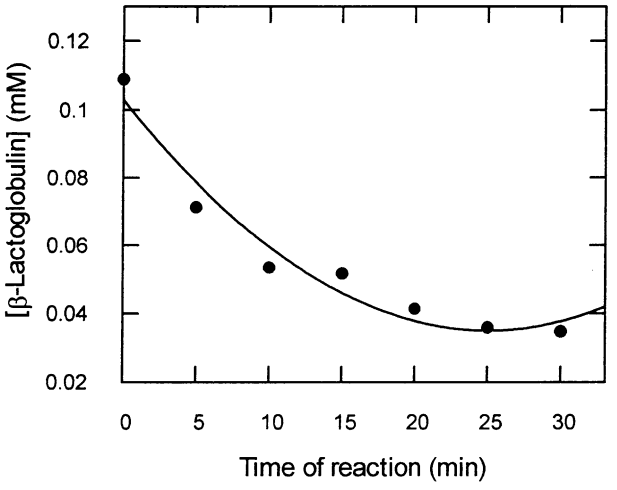


Fig. 2. Disappearance of β-lactoglobulin B, initial concentration of 2.0 mg/ml, during proteolysis by trypsin at 250 MPa and 25.0 °C. The line shown is obtained by fitting a second order polynomial to the data. Reaction conditions are as described in Fig. 1.

products are negligible, will provide the basis for the following kinetic analysis of the rate data. The initial rate of hydrolysis, $v$, of β-lactoglobulin B [β-lg B], may be described by the Michaelis–Menten equation:

$$v = \frac{k_c[E]_0[\beta\text{-lg B}]}{[\beta\text{-lg B}] + K_m} \quad (1)$$

where $[E]_0$ is the initial concentration of enzyme, and $k_c$ and $K_m$ are the catalytic constant of hydrolysis and the Michaelis constant, respectively. The dependence of the initial rates on the concentration of β-lactoglobulin B at pressures of 0.1, 100, 150 and 250 MPa are shown in

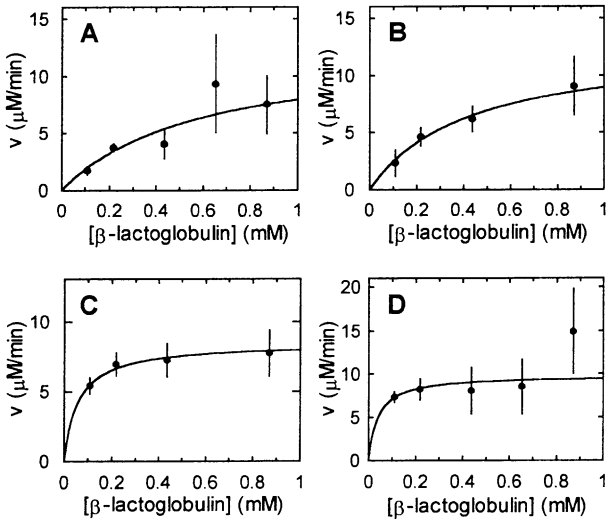


Fig. 3. Steady-state kinetics of tryptic β-lactoglobulin B hydrolysis at 0.1 MPa (A), 100 MPa (B), 150 MPa (C), and 250 MPa (D), at pH 7.6 and 25.0 °C. Initial rates of proteolysis are shown as a function of the concentration of β-lactoglobulin for various concentrations of enzyme: (A) 4.0 μM and (B)–(D) 51 nM. Results from three independent experiments for each pressure are shown. Lines shown are obtained by fitting the parameters $V_{max} = k_c[E]_0$ and $K_m$ in the Michaelis–Menten equation to the data. Error bars represent standard error of means (S.E.M.).

Fig. 3, and the values of $k_c$ and $K_m$ were calculated using data from this figure. The steady-state kinetic parameters obtained from fits of the Michaelis–Menten equation are presented in Table 1.

The pressure-dependence of the catalytic constant reveals that the proteolytic susceptibility of β-lactoglobulin B increases 60- to 80-fold in the pressure range 0.1–250 MPa. It is known that pressure may change the kinetics of enzymatic reactions, as a result of conformational changes of the substrate, leading to exposure of peptide bonds susceptible to solvent and enzyme. In the catalytic step, the tertiary structure of the enzyme will also undergo structural rearrangements in order to accommodate the cleavage site of the substrate towards the active site of the enzyme, which will be followed by adjustment of solvation until the enzyme–substrate complex reaches the transition state. Such processes will lead to significant volume changes in the transition state involved in the enzymatic hydrolysis of the substrate, compared with the enzyme–substrate complex. In accordance with transition state theory, the observed overall volume change is equal to the volume change of the rate determining step, i.e. the activation volume as defined by the difference between the volume of the activated state and the volume of the Michaelis–Menten complex. The effect of pressure on the tryptic hydrolysis of β-lactoglobulin corresponds to a significant decrease in volume of the activated state, as indicated by the 60- to 80-fold increase of the catalytic rate constant in the pressure range investigated. However, the effect of pressure on β-lactoglobulin is maximal at 100 MPa, where increasing pressure presumably promotes exposure of normally hindered cleavage sites in native β-lactoglobulin B. It is known that trypsin is not inactivated by pressure at pressure below 300 MPa (Stapelfeldt et al., 1996); thus, the pressure maximum is not due to enzyme denaturation. For digestion of β-lactoglobulin by thermolysin, Dufour et al. (1995) observed pressure-induced changes in the susceptibility to hydrolysis of different peptide bonds in β-lactoglobulin B, as seen from changes of the resultant peptide profiles. It indicated a combined influence of pressure-induced activation of thermolysin and change of enzyme specificity with a partial unfolding of β-lactoglobulin at neutral pH. However, it was not possible to identify the dominating factor. It might further be argued that formation of disulfide bonds, resulting in aggregation of individual β-lactoglobulin molecules, may protect certain peptide sequences towards the enzyme, in effect yielding other peptide profiles (Knudsen, Olsen, Skibsted, & Otte, in preparation).

The enhanced digestibility of β-lactoglobulin B at elevated pressure, due to an increased catalytic constant, was further accentuated by a decreasing Michaelis constant, indicating that pressure denaturation of β-lactoglobulin B also resulted in changes in the protein conformation that facilitated binding of the substrate to the enzyme. Interpretation of the pressure effect on $K_m$ can be made according to an ordinary single-step equilibrium;

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \{E - S\} \overset{k_2}{\rightarrow} E + P \quad (2)$$

if the usual assumption, that $k_2 \ll k_{-1}$ is adopted (Morild, 1981). In this case, $K_m$ equals $K_s$, the dissociation constant of the Michaelis complex, and the binding volume can be interpreted as a simple association volume (Morild, 1981):

$$E + \beta\text{-lg B} \overset{\Delta V_{bind}}{\rightleftarrows} \{E{-}\beta\text{-lg B}\} \quad (3)$$

Table 1
Influence of pressure on the steady-state kinetic parameters for hydrolysis of β-lactoglobulin B by trypsin in aqueous solution with pH 7.6 and ionic strength 0.08 (NaCl) at 25.0 °C, together with reaction volume for binding of β-lactoglobuIin B to the enzyme, as derived from $K_m$ in Fig. 4

| $P$ (MPa) | $k_c$ ($s^{-1}$) | $K_m$ (mM) | $k_c/K_m$ ($M^{-1}$ $s^{-1}$) | $\Delta V_{bind}$ (ml $mol^{-1}$) |
|---|---|---|---|---|
| 0.1 | 0.05±0.006 | 0.6±0.4 | 83±56 | |
| 100 | 4.2±0.8 | 0.42±0.14 | 10,000±3800 | |
| 150 | 2.8±0.14 | 0.06±0.01 | 46,700±8100 | −37.2±19 |
| 250 | 3.2±0.05 | 0.04±0.02 | 80,000±42,000 | |

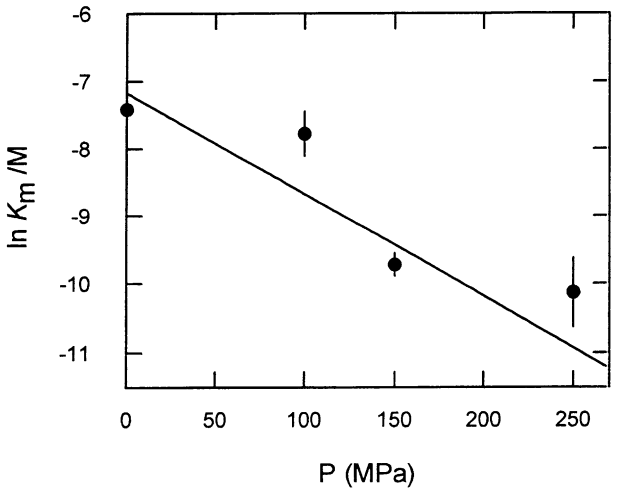


Fig. 4. Pressure dependency of the Michaelis constant, $K_m$, for the binding of β-lactoglobulin B to trypsin at pH 7.6 and 25.0 °C, analyzed according to $\ln K_m = \Delta V_{bind} P/RT + \text{constant}$. Experimental values were taken from Table 1. Error bars represent standard error of means (S.E.M.). For equation, see text.

The relationship between $\Delta V_{bind}$ and $K_m$ is thus given by:

$$\left(\frac{\partial \ln K_m}{\partial P}\right)_T = \frac{\Delta V_{bind}}{RT} \quad (4)$$

The dependency of $K_m$ for binding of β-lactoglobulin B to trypsin, as a function of pressure, is shown in Fig. 4, yielding a binding volume, $\Delta V_{bind}$ of $-37$ ml mol$^{-1}$ for the reaction of Eq. (3), for which an enthalpy change $\Delta H_{bind} = -28$ kJ mol$^{-1}$ was previously determined at ambient pressure (Olsen et al., 2000).

The overall catalytic efficiency of substrate conversion, as defmed by $k_c/K_m$, was significantly intensified at high pressure as a consequence of the linear combination of the volume changes of the association process and the catalytic step. Thus, the substrate specificity of β-lactoglobulin B increases as much as 1000-fold between ambient pressure and 250 MPa (Table 1), showing that high pressure treatment of whey concentrate should be considered as a process for reduction of allergenicity of whey products. This can be visualized by considering the content of β-lactoglobulin normally present in bovine milk (0.3%), for which 63 min at 25 °C is required for hydrolysis applying 4 μM of trypsin. Elevating the pressure to 250 MPa reduces the required time to 16 s, assuming, in both cases, that steady-state conditions prevail during the hydrolysis.

Three steps were previously identified in the pressure denaturation of β-lactoglobulin, including an initial pressure-melting around 50 MPa, followed by a reversible unfolding, with exposure of hydrophobic regions (half denaturation at 123 MPa), and irreversible denaturation with thiol-disulfide exchanges at higher pressures (above approximately 300 MPa; Stapelfeldt & Skibsted, 1999). It is interesting to note that the effect of pressure on the catalytic constant is seen in the pressure region up to 100 MPa, where β-lactoglobulin is pressure-melted with partial collapse of the inner calyx. Exposure of hydrophobic regions during the subsequent reversible pressure-denaturation has only minor effects on the catalytic constant, whereas binding of β-lactoglobulin B to the enzyme is further enhanced. The volume of binding is $-37$ ml mol$^{-1}$, a volume somewhat smaller than the volume of reversible denaturation, $\Delta V^\circ = -73$ ml mol$^{-1}$ (Stapelfeldt et al., 1996). A more detailed interpretation of these changes in relation to solvation will depend on determination of partial molar volumes of the involved species and such work is in progress.

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