Steady-State Kinetics and Thermodynamics of the Hydrolysis of β-Lactoglobulin by Trypsin

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Hydrolysis of β-lactoglobulin (in an equimolar mixture of the A and B variant) by trypsin in neutral aqueous solution [pH 7.7 at 25 °C, ionic strength 0.08 (NaCl)] was followed by capillary electrophoresis and thermodynamic parameters derived from a Michaelis−Menten analysis of rate data obtained at 10, 20, 30, and 40 °C for disappearance of β-lactoglobulin. Enthalpy of substrate binding to the enzyme and the energy of activation for the catalytic process were found to have the values, $\Delta H_{bind} = -28 \pm 4$ kJ mol^{-1} and $E_a = 51 \pm 18$ kJ mol^{-1}, respectively. Thus, β-lactoglobulin shows an enthalpy of activation for free substrate reacting with free enzyme of about 21 kJ mol^{-1}, corresponding to a transition state stabilization of 60 kJ mol^{-1} when compared to acid-catalyzed hydrolysis. The catalytic efficiency of trypsin in hydrolysis of β-lactoglobulin is increased significantly by temperature; however, this effect is partly counteracted by a weaker substrate binding resulting in an increase by only 25%/10 °C in overall catalytic efficiency.

Keywords: *β-Lactoglobulin; trypsin; hydrolysis; steady-state kinetics; thermodynamics*

The dominant whey protein in bovine milk, β-lactoglobulin, has been well-characterized by both physical and chemical methods, but its physiological and nutritive function is not fully understood. Several genetic variants have been identified, and the major variants are β-lactoglobulin A and B that are both composed of 162 residues differing only at positions 64 and 118 (Swaisgood, 1982). β-Lactoglobulin shows a remarkable stability at low pH, resisting denaturation at pH 2 (Aschaffenburg and Drewry, 1957) and pepsin hydrolysis even at the optimum pH for this hydrolytic enzyme (pH 2.5) (Schmidt and van Markwijk, 1993; Stapelfeldt et al., 1996). In some cases, this resistance leads to diet-dependent secretion of β-lactoglobulin antigens in maternal milk, indicating that β-lactoglobulin is able to pass across membrane interfaces. However, at higher pH, trypsin and thermolysin are both capable of hydrolyzing β-lactoglobulin (Schmidt and Poll, 1991), thus avoiding the hyperallergenic effect of β-lactoglobulin without affecting the nutritive value of the protein.

Several structural and biophysical data available for β-lactoglobulin provide the basis for an understanding of the accessibility of proteolytic enzymes to this protein. The pH-dependent self-association equilibria of the protein are in favor of dimers below pH 3.7 and above pH 5.2 and are shifted toward the monomer as the pH is decreased below 3.0 or increased above 6.5, respectively. Between pH 3.7 and pH 5.2, a concentration-dependent reversible tetramerization occurs (Timasheff et al., 1966). The influence of pH on the structural changes of β-lactoglobulin studied by tryptic hydrolysis indicated a conformational rearrangement of β-lactoglobulin above pH 7.5 increasing the accessibility to β-lactoglobulin for trypsin (Chobert et al., 1991), probably as a result of a tyrosine moiety hidden in the interior of the protein at lower pH values becoming exposed to the solvent around pH 8 (Tanford et al., 1959).

Tryptic hydrolysis of β-lactoglobulin is thus important for producing hypoallergenic whey protein hydrolysates under conditions in which the structural integrity of the substrate and the hydrolysates obtained are conserved. Heat treatment followed by enzymatic hydrolysis of whey has been performed as a means of eliminating the allergenicity induced by β-lactoglobulin (Jost et al., 1987; Schmidt and van Markwijk, 1993). However, at substrate concentrations normally employed for the hydrolysis of whey, high-temperature treatments can lead to irreversible denaturation of the proteins. The kinetics of tryptic hydrolysis of β-lactoglobulin has been studied by several investigators (Huang et al., 1994; van Willige and Fitzgerald, 1995). However, no steady-state kinetic experiments have been performed to investigate the influence of temperature on the tryptic digestion of β-lactoglobulin and its impact on the general catalytic efficiency of substrate conversion under nondenaturating conditions.

The present study was conducted to investigate the initial proteolysis of a commercial β-lactoglobulin preparation containing the mixture of β-lactoglobulin A and B as is present in most whey powder. The steady-state kinetics and thermodynamics of the tryptic hydrolysis of β-lactoglobulin was analyzed within the Michaelis−Menten framework (i) to determine the enthalpy of binding of β-lactoglobulin to trypsin, (ii) to determine the energy of activation for the catalytic reaction, and (iii) to elucidate the transition-state stabilization of the substrate performed by the enzyme. Such a kinetic study depends on analyzing the reaction mixture at reaction times, in which only a small fraction of the substrate has reacted. Since the hydrolysis products may vary (and are not known), the hydrolysis was monitored by determining the amounts of unreacted substrate by capillary electrophoresis (CE), a technique

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successfully used for analysis of a range of proteins and complementary to liquid chromatography. In capillary electrophoresis, separation is based on charge-to-mass ratio differences of the components, and quantification can be performed in one step as in HPLC. However, the resolution potential of capillary electrophoresis is higher than for HPLC; only small volumes of samples and (generally nontoxic) buffers are needed, and additionally, capillary electrophoresis may be applied on samples more concentrated than for HPLC.

MATERIALS AND METHODS

Materials. β-Lactoglobulin from bovine milk (90% purity, containing the genetic variants A and B in equal amounts ±3% as determined by capillary electrophoresis), analytical grade tris[hydroxymethyl]aminomethane (Tris) and tosyl phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin, 10 000 BAEE U/mg) were purchased from Sigma Chemical Co (St. Louis, MO). Soybean trypsin inhibitor was from Boehringer Mannheim (D-68298 Mannheim, Germany). All other chemicals were of analytical grade, and solutions were based on highly purified water (Milli-Q Plus, Millipore Corp, Bedford, MA).

Hydrolysis Experiments. The trypsin-catalyzed hydrolysis of β-lactoglobulin was studied in a buffer consisting of 50 mM Tris, 10 mM $CaCl_2$, pH 7.7, at 25 °C and ionic strength 0.08 (NaCl). The rates of hydrolysis were measured in triplicate at five substrate concentrations of β-lactoglobulin (0.11, 0.22, 0.44, 0.66, and 0.88 mM) using enzyme concentrations of 2.5, 2.1, 0.68, and 0.98 μM, respectively, at 10, 20, 30, and 40 °C. Samples of the incubation mixtures were taken at appropriate time intervals, and the enzyme activity was quenched by addition of trypsin inhibitor to an inhibitor/enzyme molar ratio of 4:1. These samples were analyzed by capillary electrophoresis without any further preparation.

Capillary Electrophoresis. β-Lactoglobulin was quantified using a slight modification of the CE method described by Otte et al. (1994). Analysis was performed on a HP3DCE instrument (G1602A, Hewlett-Packard A/S, Waldbronn, Germany), mounted with an untreated 50 mm i.d. fused-silica capillary of 64.5 cm total length. Samples were injected hydrodynamically (3 s at 50 mbar) at the anode, and the voltage was adjusted to give a current of 35−40 μA. Separation was performed at 30 °C using 100 mM sodium phosphate, pH 2.5, as run buffer, and the absorbance was monitored at 214 nm. Quantification was based on standard curves made with varying concentrations (2−16 mg/mL) of the pure β-lactoglobulin in sample buffer.

Kinetic Analysis. The initial rate of hydrolysis was calculated from the decrease of the integrated area of the peak representing intact β-lactoglobulin as a function of time. The steady-state kinetic parameters, k_c and K_m, were determined from fits of the Michaelis−Menten equation to the initial rates of hydrolysis as a function of substrate concentration using the nonlinear least-squares fitting program GraFit (Erithacus Software, Ltd.). Values for k_c and K_m at various temperatures yield the thermodynamic parameters of the trypsin-catalyzed hydrolysis of β-lactoglobulin.

RESULTS AND DISCUSSION

Time Course of Tryptic Hydrolysis of β-Lactoglobulin. Bovine β-lactoglobulin was incubated with TPCK-trypsin, and the hydrolysis was followed by monitoring the gradual disappearance of intact β-lactoglobulin by CE as a function of time. Typical CE profiles obtained during the progress of the hydrolysis of β-lactoglobulin are shown in Figure 1 where unhydrolyzed β-lactoglobulin is represented by the peak with migration time around 13 min.

Steady-State Kinetics. As seen in Figure 2, the rate of hydrolysis decreased with time, indicating a decrease

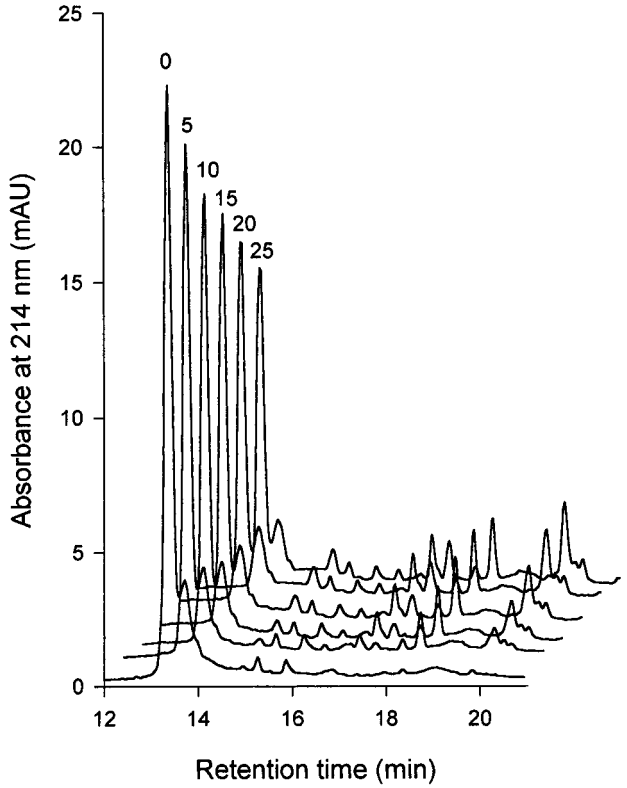

Figure 1. Capillary electropherograms of the tryptic hydrolysates of β-lactoglobulin at different times of the reaction. Unreacted β-lactoglobulin is represented by the peak area with migration time around 13 min. Reaction conditions were 50 mM Tris-10mM $CaCl_2$, pH 7.7, ionic strength 0.08 (NaCl), at 20 °C with a substrate concentration of 2 mg/mL and 2.1 μM trypsin. CE conditions are described in the Materials and Methods section.

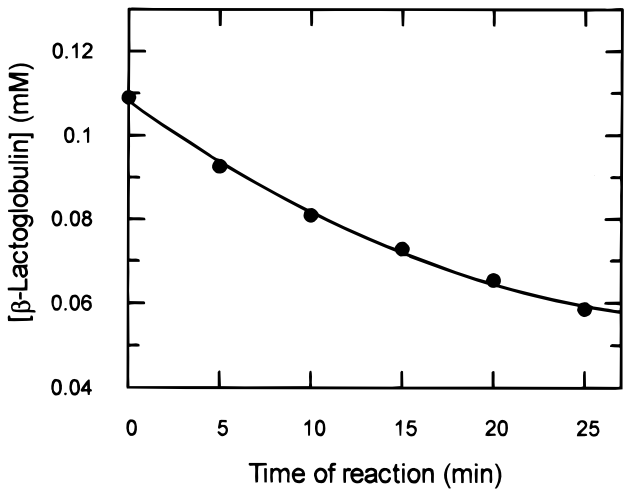

Figure 2. Hydrolysis time course of β-lactoglobulin obtained with trypsin. The line shown is obtained by fitting a second-order polynomial to the data. Reaction conditions are as described in Figure 1.

in the concentration of β-lactoglobulin and possibly an inhibiting effect of the products formed during the hydrolysis. However, kinetic data are based on the initial rates of hydrolysis determined by an extrapolation (polynomial) to time zero, thus reflecting the steady-state conditions prevailing at the start of the reaction, where the concentration of β-lactoglobulin can be assumed constant and the concentration of products are negligible, and the enzyme concentration is much

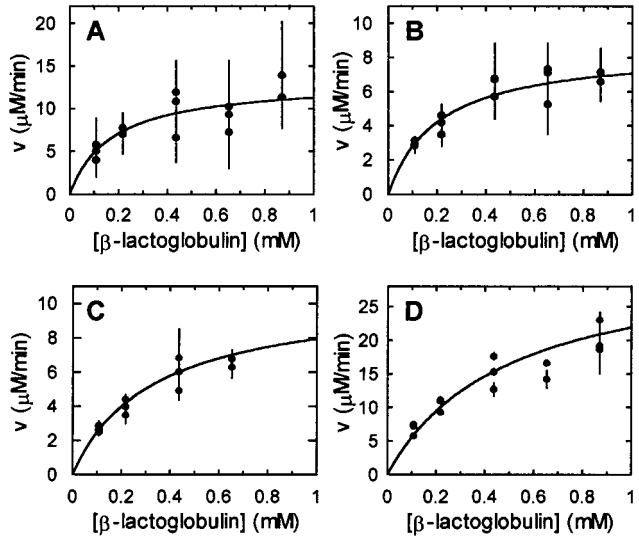

Figure 3. Steady-state kinetics of tryptic β-lactoglobulin hydrolysis at (A) 10, (B) 20, (C) 30, and (D) 40 °C, pH 7.7. Initial rates of proteolysis of β-lactoglobulin are shown as a function of the substrate concentration for various concentrations of enzyme: (A) 2.5, (B) 2.1, (C) 0.68, and (D) 0.98 μM. Results from three independent experiments are shown. Lines shown are obtained by fitting the parameters $V_{max} = k_c[E]_0$ and K_m from the Michaelis−Menten equation to the data.

Table 1. Steady-state Kinetic Parameters for Hydrolysis of β-Lactoglobulin by Trypsin in Aqueous Solution with pH 7.7 and Ionic Strength 0.08 and Energy of Activation for Catalytic Reaction and Enthalpy of Substrate Binding to the Enzyme

t (°C)	k_c (s^{-1})	K_m (mM)	k_c/K_m (M^{-1} s^{-1})	E_a (kJ mol^{-1})	ΔH_{bind} (kJ mol^{-1})
10	0.088 ± 0.007	0.17 ± 0.04	520 ± 130		
20	0.067 ± 0.005	0.20 ± 0.03	340 ± 60	50.8 ± 18.0	−27.6 ± 4.3
30	0.26 ± 0.01	0.33 ± 0.02	790 ± 220		
40	0.53 ± 0.07	0.45 ± 0.09	1180 ± 280		

smaller than the substrate concentration. Accordingly, the initial rate of hydrolysis, v, of the substrate, [S], may be described by the Michaelis−Menten equation:

$$v = \frac{k_c[E]_0[S]}{[S] + K_m}$$

where $[E]_0$ is the 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 at the four temperatures are shown in Figure 3, and the corresponding values of k_c and K_m obtained from fits of the Michaelis−Menten equation to the data are given in Table 1.

It has been found that the β-lactoglobulin variant A is more accessible to trypsin than the B variant (Huang et al., 1994; van Willige and Fitzgerald, 1995). However, the substrate specificity, as defined by k_c/K_m, is comparable for the A and B variant, implying that at equal concentrations of the two variants the proteinase will hydrolyze both variants with comparable rates since both substrates will have comparable turnover numbers for this enzyme. In the present study, the CE analysis of the hydrolysates separates the products from the starting material and intact β-lactoglobulin, and the disappearance of the substrate represents the combined consumption of β-lactoglobulin A and B. The determined parameters are accordingly of practical relevance, when a commercial β-lactoglobulin preparation, normally containing a mixture of β-lactoglobulin A and B, is hydrolyzed under industrial conditions.

The steady-state kinetic parameters found in the present study reveal that the overall catalytic efficiency of substrate conversion as defined by k_c/K_m is increased only 2-fold by a 30 °C increase of temperature. Thus, the proteolytic susceptibility of β-lactoglobulin regarding trypsin as a mean of producing hypoallergenic whey protein hydrolysates is affected only to a limited extent at higher temperatures since the increase in catalytic turnover number with temperature partly is compensated by a weaker binding of the substrate in the enzyme−substrate complex as the temperature is increased. However, the content of β-lactoglobulin normally present in milk (0.3%) may be digested by trypsin within 6 min at 10 °C or 2 min at 40 °C if one applies 10 mM of enzyme assuming that steady-state conditions prevail during hydrolysis.

The catalytic mechanism of enzymes may be explained by the transition state theory (Fersht, 1985), which relates the activation energy for the enzymatic conversion of substrate to products, i.e., the difference in free energy between the free enzyme and substrate and the transition state, to the sum of the energy released upon binding of substrate to enzyme in the most stable complex (Michaelis-complex) and the energy associated with bringing the enzyme−substrate complex from the ground state to the transition state.

In Michaelis−Menten kinetics, K_m represents the apparent dissociation constant of all enzyme-bound species. In the simple case with β-lactoglobulin illustrated by the reaction of eq 1, K_m equals K_s, the dissociation constant of the Michaelis-complex:

$$E + \beta\text{-lactoglobulin} \underset{K_m}{\overset{\Delta G_{bind}}{\rightleftharpoons}} \{\beta\text{-lactoglobulin-E}\} \quad (1)$$

and the relationship between ΔG_{bind} and K_m is thus given by $\Delta G_{bind} = RT \ln K_m$. Since K_m in this case is an equilibrium dissociation constant, the temperature dependency can be analyzed according to the van't Hoff equation, $d \ln K_m / d(1/T) = \Delta H_{bind}/R$, where ΔH_{bind} is the reaction enthalpy of the binding process. The dependency of K_m for binding of β-lactoglobulin to trypsin as a function of temperature is shown in Figure 4A yielding a reaction enthalpy, ΔH_{bind} of −28 kJ mol^{-1} for the reaction of eq 1.

The free energy, $\Delta G^{\ddagger}$, associated with bringing the Michaelis-complex from the ground state to the transition state, illustrated by the reaction of eq 2,

$$\{\beta\text{-lactoglobulin-E}\} \underset{\Delta G^{\ddagger}}{\overset{k_c}{\longrightarrow}} \{\beta\text{-lactoglobulin-E}\}^{\ddagger} \quad (2)$$

can be analyzed according to the Arrhenius equation. Figure 4B shows the temperature dependence of the catalytic constant, k_c, for the hydrolysis of β-lactoglobulin yielding an energy of activation, E_a, of 51 kJ mol^{-1} for the reaction of eq 2, which may be converted to $\Delta H^{\ddagger}$ ($= E_a - RT$).

In accordance with transition state theory, the reactions of eqs 1 and 2 may be combined to the reaction of eq 3:

$$E + \beta\text{-lactoglobulin} \underset{\Delta G_T^{\ddagger}}{\overset{k_c/K_m}{\rightleftharpoons}} \{\beta\text{-lactoglobulin-E}\}^{\ddagger} \quad (3)$$

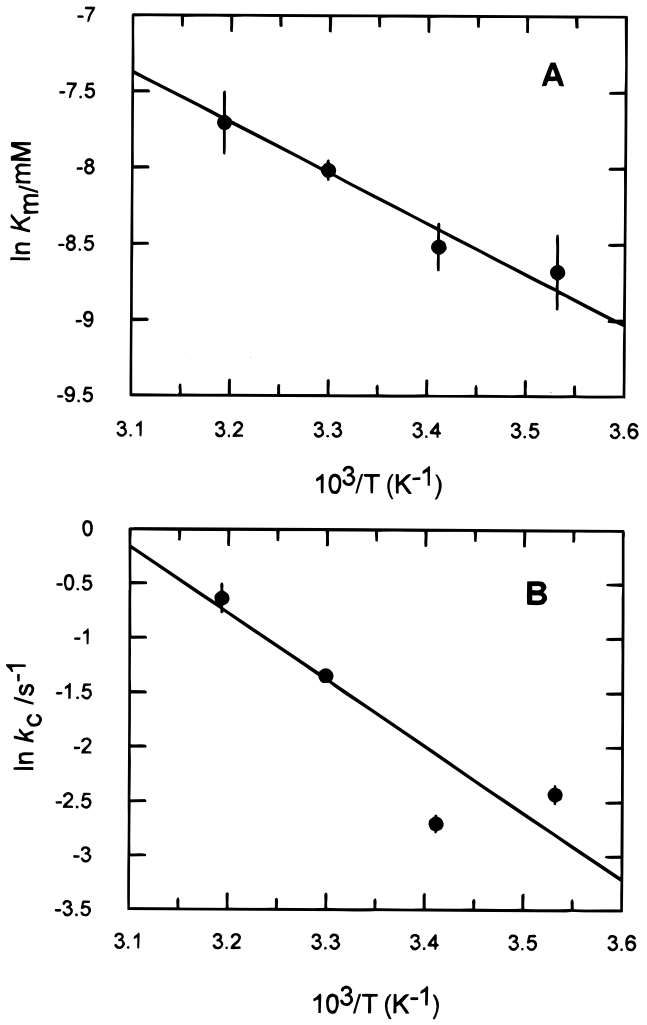

Figure 4. Temperature dependencies of (A) the Michaelis-constant, K_m, for the binding of β-lactoglobulin to trypsin at pH 7.7 analyzed according to the van't Hoff equation for the reaction of eq 1 and of (B) the catalytic constant, k_c, for the hydrolysis of β-lactoglobulin at pH 7.7 analyzed according to the Arrhenius equation for the reaction of eq 2.

where $\{\beta$-lactoglobulin-E$\}^{\ddagger}$ is the transition state complex of the enzyme−substrate. Thus, the substrate specificity k_c/K_m (in M^{-1} s^{-1}) corresponding to the apparent second-order reaction of eq 3 has an enthalpy of activation, which may be calculated from eq 4

$$\Delta H_T^{\ddagger} = (E_a - RT) + \Delta H_{bind} \quad (4)$$

to have a value of 21 kJ mol^{-1}. For comparison, the enthalpy of activation for acid-catalyzed hydrolysis of a typical peptide bond is approximately 80 kJ mol^{-1} (Long and Truscott, 1963).

In conclusion, it has been shown that tryptic hydrolysis of β-lactoglobulin in neutral solution is enhanced by lowering the energy barrier by some 60 kJ mol^{-1} when compared to acid-catalyzed hydrolysis. This dramatic transition state stabilization is, however, partly counteracted by a relatively weak binding of β-lactoglobulin to the enzyme at this pH. Although, temperature has a limited impact on the hydrolysis of β-lactoglobulin at neutral pH as the result of a balance between two counteracting effects, the information presented in this study will be of value to the dairy industry in optimizing process parameters for production of hypoallergenic whey protein hydrolysates. The study should also enhance the understanding of the kinetics and thermodynamics of β-lactoglobulin hydrolysis.

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