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Revisiting the enzymatic kinetics of pepsin using isothermal titration calorimetry

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ABSTRACT

Pepsin is the first protease that food proteins encounter in the digestive tract. However, most of the previous studies on the enzymatic kinetics of pepsin were based on the hydrolysis of small synthetic peptides, due to the limitations in methodology and the complexity of protein substrate. To better understand the role of pepsin in protein digestion, we used isothermal titration calorimetry to study the enzymatic kinetics of pepsin with bovine serum albumin as the substrate. We found that pepsin has a higher catalytic rate at lower pH, while its affinity to substrate is lower. At the same pH, pepsin has lower activity and affinity at higher ionic strengths. We found contrasting kinetic parameters for pepsin-catalyzed hydrolysis of bovine serum albumin and of small synthetic peptides. Time-dependent kinetics also showed that pepsin has lower efficiency towards intermediate peptides during hydrolysis.

1. Introduction

The digestion of food proteins in the gastrointestinal tract has been studied both *in vivo* and *in vitro* for decades. These studies have investigated the nutritional value (Boirie et al., 1997; Nilsson, Holst, & Björck, 2007), digestibility (Hsu, Vavak, Satterlee, & Miller, 1977; Denis et al., 2016), and allergenicity (Schmidt, Meijer, Slangen, & van Beresteijn, 1995; Kopper et al., 2004; Polovic et al., 2007) of proteins, however, the reaction kinetics of protein digestion has not been fully quantified to date. The digestion of protein starts in the stomach, where pepsin is the major enzyme present. Therefore, the enzymatic kinetics of pepsin is an important aspect of the digestion process of food proteins that requires better understanding.

Pepsin (EC 3.4.23.1) is an aspartic acid protease. Its zymogen, pepsinogen is secreted by the chief cells on gastric mucosa, and activated in low pH (Kageyama, 2014). Pepsin has 330–350 amino acid residues with mainly β-sheets. The catalytic site of pepsin is located in the middle narrow slit of two lobes; two aspartic acid residues, Asp32 and Asp215, are located at the end of each domain (Dunn, 2001). These two aspartic acid residues are connected through a low-barrier hydrogen bond. Findings from recent decades suggested that this low-barrier hydrogen bond is the key feature in the catalytic mechanism for aspartic proteases, which facilitates proton transfer during catalysis (Northrop, 2001; Dunn, 2002).

The specificities of proteinases are often characterized by the cleaved peptide bond (P1-P1'), which consists of two amino acid residues. However, the substrate binding and specificity may also involve the amino acid residues on either side of the cleaved peptide bond (Powers, Harley, & Myers, 1977; Foltmann, 1981):

$$\begin{array}{lccccccccccccc} \textit{Substrate} & P_4 & — & P_3 & — & P_2 & — & P_1 & \downarrow & P_1' & — & P_2' & — & P_3' & — & P_4' \\ \textit{Enzyme} & S_4 & & S_3 & & S_2 & & S_1 & & S_1' & & S_2' & & S_3' & & S_4' \end{array}$$

where the individual amino acid residues on the substrate are designated P1, P2, P1′, etc. and the corresponding subsites of the enzyme are S1, S2, S1′, etc. The arrow indicates the peptide bond cleavage site between the P1 and P1′ residues.

The specificity of pepsin (i.e. the cleavage probability of peptide bonds by pepsin) is mainly influenced by the amino acid residues at position P1 and P1′, while the amino acid residues at other subsites may also play a role. In general, pepsin prefers to cleave after phenylalanine, leucine, and methionine, whereas it rarely cleaves after histidine, lysine, proline, and arginine. Aromatic residues tyrosine, tryptophan, and phenylalanine are favored at position P1′. Proline is strongly disfavored at P2, P2′, and P3′ position, while histidine, lysine, and arginine are disfavored at the P3 position. The P4 and P4′ positions have little influence. (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008; Ahn, Cao, Yu, & Engen, 2013).

The influence of pH on enzyme activity is generally recognized. Pepsin, as an aspartic protease, has a very low pH optimum. Kondjoyan, Daudin, and Santé-Lhoutellier (2015) studied the digestibility of myofibrillar proteins and found maximum pepsin activity at pH around 2.

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Pletschke, Naudé, and Oelofsen (1995) studied the hydrolysis of hemoglobin by porcine and ostrich pepsin, and also found an optimum pH of 2.0 for pepsin activity.

In contrast to the influence of pH, the effect of ionic strength on enzyme activity is less often discussed. Previous work by Dale and White (1983) showed that increasing the ionic strength clearly decreases the reaction rate of immobilized pancreatic ribonuclease, while Butré, Wierenga, and Gruppen (2012) studied the enzymatic hydrolysis of whey protein isolate by alcalase and neutrase, and also found that the presence of 0.5 M NaCl decreased the rate of hydrolysis.

The enzymatic kinetics can be described by the Michaelis-Menten model (Michaelis & Menten, 1913; Johnson & Goody, 2011):

$$v = \frac{k_{cat} E S}{K_m + S} \tag{1}$$

where v is the rate of the enzymatic reaction, E is the enzyme concentration, and S is the substrate concentration. In this equation, K_m is the Michaelis constant (defined as the substrate concentration where half of the maximum reaction rate is reached) that can describe the binding affinity between enzyme and substrate, and k_{cat} is the catalytic constant, also known as the turnover number. It is the maximum amount of substrate converted to product per enzyme molecule per second. k_{cat}/K_m is defined as the specificity constant that indicates the catalytic proficiency of an enzyme. k_{cat} and k_{cat}/K_m are now recognized as the two primary steady-state kinetic parameters for enzymes (Miller & Wolfenden, 2002).

To obtain the kinetic data of the enzymatic reaction, spectrophotometric methods are commonly used. Since spectrophotometric methods require specific chromophores on either the substrate or product, they are constrained by substrate properties and reaction conditions (temperature and pH), and are often laborious. Alternatively, calorimetric methods can be used to measure reaction rate by monitoring the enthalpy change, since the enthalpy change is ubiquitous in most enzymatic reactions.

Isothermal titration calorimetry (ITC) is one of the calorimetric methods that use power compensation to keep reaction conditions isothermal. As a chemical reaction takes place in the measurement cell, the thermal power from the controlled heater is adjusted according to the enthalpy change of the reaction (Freyer & Lewis, 2008). The monitored thermal power is directly proportional to the reaction rate, which simplifies data analysis. Reaction rates at different substrate concentrations can be obtained in a single experiment by subsequently injecting the substrate (under pseudo-first-order conditions), rather than requiring multiple experiments. Moreover, low amounts of enzyme and substrate are required due to the high sensitivity of ITC (Todd & Gomez, 2001).

Pepsin is the first protease that food proteins encounter in the digestive tract, thus the usual substrate for pepsin is mostly intact proteins. However, most previous studies on the enzymatic kinetics of pepsin were based on the hydrolysis of small synthetic peptides. Most studies on pepsin-catalyzed hydrolysis of proteins did not quantify the kinematic parameters. The enzymatic kinetics of pepsin with intact protein is most relevant for realistic situations, rather than with short peptides. With information on the kinetics of pepsin in realistic situations, we could better understand the role of pepsin in the digestion process of food proteins. Therefore, we aim to study the enzymatic kinetics of pepsin with bovine serum albumin as a substrate, using ITC, taking the effect of pH and ionic strength into consideration. We hypothesize that both the affinity and efficiency of pepsin are influenced by pH and ionic strength, and the physiological role of pepsin is related to food disintegration.

2. Materials and method

2.1. Materials

Pepsin from porcine gastric mucosa (lyophilized powder, 3200–4500 units per mg protein, MW = 34.6 kDa), bovine serum albumin (purity ⩾ 98%) from bovine milk, and all other chemicals used were purchased from Sigma Aldrich (St. Louis, MO, USA). All of the water used in the experiments was obtained from a Milli-Q Integral Water Purification System (Merck Millipore, Billerica, MA, USA).

2.2. Sample preparation

Buffers at different pH and ionic strength were prepared. 100 mM phosphate buffer at pH 2, pH 2.5, pH 3, and pH 3.5 were used. At pH 2, three phosphate buffers were prepared at the ionic strength of 43 mM (that of the buffer without additional salt), 89 mM (equivalent to the total ionic strength of the electrolytes in the simulated gastric fluid according to the international consensus by Minekus et al. (2014)) and 154 mM (physiological salt condition). The ionic strength of buffers at pH 2.5 and pH 3 were 89 mM, and 96 mM at pH 3.5, so that the effect of pH can be compared at similar ionic strength. The ionic strengths were calculated via the buffers' ionic compositions:

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \tag{2}$$

where c_i is the molar concentration of ion i, z_i is the charge number of that ion, and the sum is taken over all ions in the solution. The ionic strengths were adjusted by adding sodium chloride when necessary. At pH 3.5, the native ionic strength of the 100 mM phosphate buffer is 96 mM.

A 10 μM stock solution of pepsin was prepared in the buffers, stored at −20 °C for up to one month, and unfrozen before use. Substrate solutions were freshly prepared. Due to the high concentration of the substrate solutions, the pH was slightly changed from the buffer pH. Thus, we adjusted the pH to avoid the effects of extra heat from a difference in pH. Before the solution was transferred to the instrument, a de-gassing step is performed with an ultrasonic device at 80 kHz for 10 min.

2.3. Isothermal titration calorimetry

The theoretical basis of determining enzymatic rate kinetics using ITC has been described previously (Morin & Freire, 1991; Williams & Toone, 1993; Todd & Gomez, 2001; Hansen, Transtrum, Quinn, & Demarse, 2016). A summary will be given below: the enthalpy change for the reaction, which is equal to the compensated thermal power (dQ/dt), is directly proportional to the rate of the chemical reaction:

$$\frac{dQ}{dt} = \Delta H_{app} V \frac{dP}{dt} \tag{3}$$

where ΔH_{app} is the apparent molar enthalpy change for the reaction, V is the volume of the calorimetric cell, and P is the product concentration. dP/dt equals to the reaction rate v, therefore Eq. (3) can be rearranged to:

$$v = \frac{1}{V \Delta H_{app}} \times \frac{dQ}{dt} \tag{4}$$

Reaction rates at different substrate concentrations can be obtained by subsequently injecting the substrate into the cell (this method is later referred as the multiple injection method). The subsequent injections are made when the reaction has reached a steady state, while significant reaction of the substrate has not yet occurred. Thus, these determined reaction rates can be regarded as the initial rates to be used in the Michaelis-Menten model (Eq. (1)). In the multiple injection method,