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The function of the milk-clotting enzymes bovine and camel chymosin studied by a fluorescence resonance energy transfer assay[1]

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ABSTRACT

Enzymatic coagulation of bovine milk can be divided in 2 steps: an enzymatic step, in which the Phe105-Met106 bond of the milk protein bovine κ-casein is cleaved, and an aggregation step. The aspartic peptidases bovine and camel chymosin (EC 3.4.23.4) are typically used to catalyze the enzymatic step. The most commonly used method to study chymosin activity is the relative milk-clotting activity test that measures the end point of the enzymatic and aggregation step. This method showed that camel chymosin has a 2-fold higher milk-clotting activity toward bovine milk than bovine chymosin. To enable a study of the enzymatic step independent of the aggregation step, a fluorescence resonance energy transfer assay has been developed using a peptide substrate derived from the 98–108 sequence of bovine κ-casein. This assay and Michaelis-Menten kinetics were employed to determine the enzymatic activity of camel and bovine chymosin under milk clotting-like conditions (pH 6.65, ionic strength 80 m*M*). The results obtained show that the catalytic efficiency of camel chymosin is 3-fold higher than bovine chymosin. The substrate affinity and catalytic activity of bovine and camel chymosin increase at lower pH (6.00 and 5.50). The glycosylation of bovine and camel chymosin did not affect binding of the fluorescence resonance energy transfer substrate, but doubly glycosylated camel chymosin seems to have slightly higher catalytic efficiency. In the characterization of the enzymes, the developed assay is easier and faster to use than the traditionally used relative milk-clotting activity test method.

Key words: fluorescence resonance energy transfer assay, Michaelis-Menten, coagulant, milk clotting

INTRODUCTION

Enzymatic coagulation of bovine milk (milk clotting) is a 2-step process. First, an enzymatic step occurs, in which the amphiphilic protein κ-CN, located on the surface of the casein micelles, is cleaved at the Phe105-Met106 peptide bond or nearby peptide bonds releasing the hydrophilic C-terminal part. This leads to the second step, in which the micelles aggregate and form a gel that eventually separates into curd and whey. Enzymes catalyzing the first step, referred to as coagulants, are used in industrial cheese production. The best enzymes for this process have a high proteolytic activity toward the Phe105-Met106-bond and a low proteolytic activity toward other bonds (Fox and McSweeney, 1998). At present, the majority of the world's cheese production is based on bovine milk and the best-known coagulants for this purpose are the aspartic peptidases, bovine and camel chymosin (EC 3.4.23.4).

Traditionally, the milk-clotting activity of chymosin is determined by a standardized assay, the relative milk-clotting activity test (**REMCAT**; International Dairy Federation, 2007), in which coagulant is added to a solution of skim milk powder with a pH and ionic strength similar to the conditions for cheese making. By comparing the time required to clot the milk with a standard, the milk-clotting activity is calculated. This procedure is time consuming and requires trained personnel. Furthermore, the assay requires comparison to an internal standard, which is subject to autodegradation and gives only one data point, the clotting time. In addition, the assay does not distinguish between the enzymatic and aggregation step, which makes it impossible to perform a more detailed analysis of the kinetics of different enzymes.

Kappeler et al. (2006) showed that camel chymosin has a significantly higher proteolytic activity toward the Phe105-Met106 bond of bovine κ-CN and a lower

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general proteolytic activity compared with bovine chymosin. Both enzymes consist of 323 AA and share 85% sequence identity. The experimentally determined isoelectrical point values are 4.8 and 5.4 for bovine and camel chymosin, respectively. A structural investigation of the 2 enzymes showed that they differ significantly with respect to their surface charge, domain flexibility, binding cleft, and conformation of the N terminus, and that glycosylation affects their milk-clotting activity (Langholm Jensen et al., 2013). To acquire a detailed understanding of the effect of the structural differences on the reaction steps in cheese making, a simplified assay that focuses on the enzymatic reaction itself was developed.

The residues around the Phe105-Met106 bond of κ-CN (residues 98–112; Figure 1) are important for substrate binding, as demonstrated previously for bovine chymosin (Visser et al., 1976; Visser et al., 1987; Gustchina et al., 1996), and therefore a suitable substrate for studies of chymosin kinetics. Kappeler et al. (2006) compared bovine and camel chymosin using residues 98–108 of κ-CN with Phe105 replaced by para-nitrophenylalanine. This chromogenic substrate allowed the reaction to be followed in real time. Unfortunately, the nitro-group affects substrate binding, which limits the usefulness of this substrate (Fox et al., 2000).

Assays based on fluorescence resonance energy transfer (**FRET**) have been successfully employed in the study of peptidases (Förster, 1948; Lakowicz, 2006). The use of a fluorophore-quencher pair makes the assay much more sensitive than standard colorimetric assays, and the fluorophore and quencher can be positioned further away from the active site of the enzyme, which lowers the risk of interfering with substrate binding. The FRET technique has been used in the design of assays for several peptidases with varying substrates, fluorophores, and quenchers (Latt et al., 1972; Matayoshi et al., 1990; Holskin et al., 1995; Grahn et al., 1998; Neumann et al., 2004; Konstantinidis et al., 2007).

In the current paper, we present a real-time assay based on the FRET technique using a labeled peptide substrate derived from the residues 98–112 sequence of bovine κ-CN (Figure 1). The assay is used to determine Michaelis-Menten kinetics of coagulants under milk-clotting conditions to examine the enzymatic step of milk clotting (i.e., cleavage of κ-CN). Furthermore, the assay enabled us to study of the enzyme kinetics of the different glycosylated forms of bovine and camel chymosin at different pH values.

MATERIALS AND METHODS

Enzyme Preparation

The kinetic study was carried out using an unglycosylated and single-glycosylated variant of bovine chymosin, and an unglycosylated and double-glycosylated variant of camel chymosin. The bovine and camel chymosin variants were isolated from the commercial products Chy-MAX Plus and Chy-MAX M

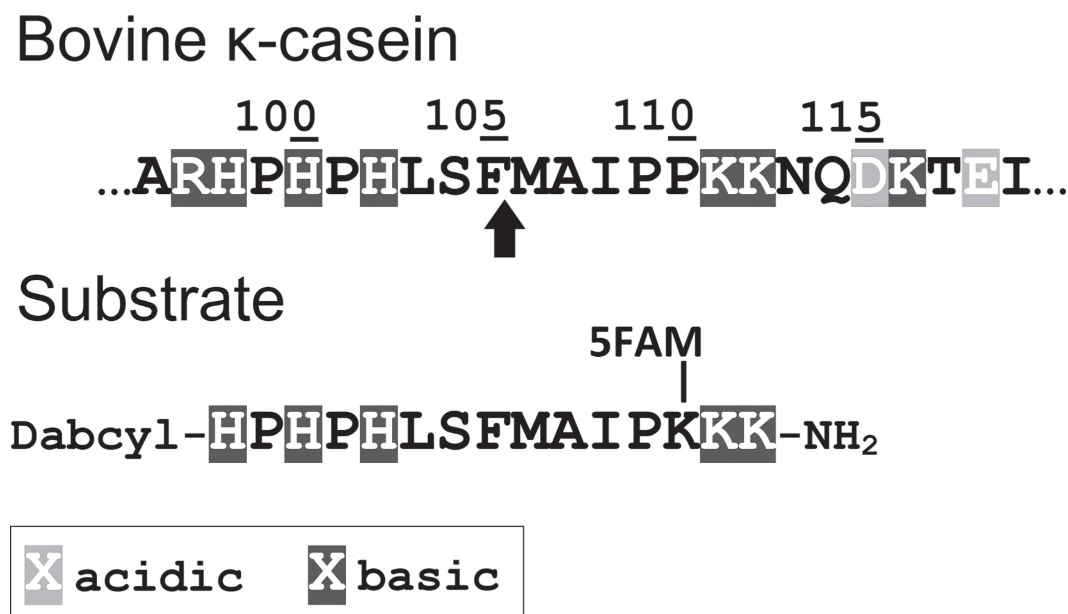

Figure 1. Illustration of the sequence of bovine κ-CN, which is cleaved by chymosin. The substrate, based on this sequence, is shown with the attached fluorophore-quencher pair. 5FAM = 5-carboxyfluorescein; Dabcyl = 4-(dimethylaminoazo)benzene-4-carboxylic acid. Color version available online.

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