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 mM). 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 under milk clotting-like conditions (pH 6.65, ionic strength 80 mM). 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.

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 peptide bond or nearby peptide bonds releasing the hydrophilic C-terminal part. 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
general proteolytic activity compared with bovine chymosin. Both enzymes consist of 323 AA and share 85% sequence identity. The experimentally determined isoelectric 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, 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-nitro-phenylalanine. 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 cloting (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

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**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.
(provided by Chr. Hansen A/S, Hørsholm, Denmark) by hydrophobic interaction chromatography with a Phenyl Superose column from Pharmacia following the procedure described previously (Langholm Jensen et al., 2013) with minor modifications. The binding buffer used for camel chymosin was 12% (wt/vol) NaCl, 0.5 M Na$_2$SO$_4$, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 6.0. The elution buffer was 20 mM MES and buffer pH 6.0. The Na$_2$SO$_4$ and MES buffer were added to the commercial chymosin product [containing 12% (wt/vol) NaCl] to match the binding buffer were added to the commercial chymosin product [containing 12% (wt/vol) NaCl] to match the binding buffer. Aliquots of the separated variants were stored at −18°C after addition of polyoxyethylene (23) lauryl ether (Brij35; Merck, Hohenbrunn, Germany) and glycerol to the separated variants to a final concentration of 0.01% (wt/vol) and 20% (vol/vol), respectively. Bovine chymosin variants were separated following the same procedure as described previously, except that the Bovine chymosin variants were separated following the same procedure as described previously, except that the 

**Kinetic Assay**

The FRET peptide Dabcyl-HPHPHLSMAIPK(5-FAM)KK-NH$_2$ (98% purity; Figure 1) was purchased from CPC Scientific (Sunnyvale, CA). Both Dabcyl, 4-(dimethylaminoazo)benzene-4-carboxylic acid, and 5-carboxyfluorescein (5-FAM) were purchased from Sigma-Aldrich (St. Louis, MO). The high-throughput assay was carried out with a black Non-Treated F96 Microtiter plate from Nunc (Thermo Fisher Scientific, Waltham, MA). The volumes are listed in Table 1. The addition of DMSO necessitated adjustment of the pH, approximately half a unit down, to the final pH values (5.50, 6.00, and 6.65). Substrate solutions were visually inspected for precipitate. The quenching efficiency was measured at each pH using a 100-μL solution of 0.1 μM enzyme. The buffer was the McIlvaine’s buffer system (McIlvaine, 1921) with 10% (vol/vol) DMSO, 0.01% (wt/vol) Brij35, and 0.02% (wt/vol) sodium azide. The ionic strength was fixed at 80 mM by supplementing with NaCl. For each pH value (5.50, 6.00, and 6.65) the buffer composition was calculated using the Buffer Maker software (Chem-Buddy, BPP, Marki, Poland). The volumes are listed in Table 1. The addition of DMSO necessitated adjustment of the pH, approximately half a unit down, to the final pH values (5.50, 6.00, and 6.65). The pH value did not change during the experiment (results not shown).

**Determination of Solubility and Quenching Efficiency**

The solubility of the substrate was measured at each pH value (5.50, 6.00, and 6.65). Substrate solutions were centrifuged using a table-top centrifuge at 12,000 × g and room temperature for 5 min, and the tubes were visually inspected for precipitate. The quenching efficiency was measured at each pH using a 100-μL solution of 0.1 μM substrate. The signal was measured as described for the FRET assay. After this, 0.2 μL of commercial camel chymosin (final enzyme concentration used in the assay (results not shown).

**Table 1.** Buffer composition (volume, mL) for making 100 mL of McIlvaine’s buffer with an ionic strength of 80 mM and an assay temperature of 32°C$^1$

<table>
<thead>
<tr>
<th>pH</th>
<th>Component</th>
<th>Phosphate</th>
<th>Citrate</th>
<th>NaCl</th>
<th>Brij35</th>
<th>DMSO</th>
<th>Azide</th>
<th>Water</th>
<th>pH (23°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.65</td>
<td>Phosphate</td>
<td>2.00</td>
<td>0.42</td>
<td>2.06</td>
<td>2.00</td>
<td>10.00</td>
<td>0.40</td>
<td>83.12</td>
<td>6.68</td>
</tr>
<tr>
<td>6.00</td>
<td>Phosphate</td>
<td>2.00</td>
<td>0.68</td>
<td>2.28</td>
<td>2.00</td>
<td>10.00</td>
<td>0.40</td>
<td>82.64</td>
<td>6.02</td>
</tr>
<tr>
<td>5.50</td>
<td>Phosphate</td>
<td>2.00</td>
<td>0.85</td>
<td>2.59</td>
<td>2.00</td>
<td>10.00</td>
<td>0.40</td>
<td>82.16</td>
<td>5.51</td>
</tr>
</tbody>
</table>

$^1$For pH control, the calculated pH at 23°C is shown. Note that due to the addition of dimethylsulfoxide (DMSO) the pH values were approximately half a unit higher than expected and had to be adjusted.

$^2$Stocks used: 1 M Na$_2$HPO$_4$ (phosphate), 1 M citric acid (citrate), 1 M NaCl (NaCl), 0.5% Brij35 (Brij35, Merck, Hohenbrunn, Germany), 5% sodium azide (azide), and deionized, sterile filtrated water (water).

tion of 0.13 μM) was added, and once the signal had reached a plateau the value was noted.

Correction for Inner Filter Effects

Correction for the inner filter effects were performed using the method of Liu et al. (1999). Substrate was prepared at the concentrations used in the kinetic assay. Measurements were carried out in triplicate on 100 μL of each sample as described previously. After this, 1 μL of a solution of 20 μM 5-FAM was added to each well (final concentration = 0.2 μM), mixed with a 60-s shake (instead of 10 s), and then measured. The change in fluorescence units (FU) was divided by the concentration of 5-FAM to calculate the slope (FU/μM) for each substrate concentration and pH value.

Data Processing

An enzymatic reaction can be described as the association of an enzyme (E) and a substrate (S) to form an enzyme-substrate complex (ES), which can either dissociate again or convert the substrate to the product (P) as shown in equation 1:

\[
E + S \xrightarrow{k_f} ES \xrightarrow{k_{cat}} E + P.
\]  

where the forward rate constant \( k_f \) denotes the association rate of the enzyme-substrate complex and the reverse rate constant \( k_r \) denotes the dissociation, whereas the catalytic rate constant \( k_{cat} \) denotes the rate of conversion into product. By assuming that the concentration of the enzyme-substrate complex is constant, the initial reaction velocity \( v_0 \) is calculated as a function of the substrate concentration, \([S]\), using the Michaelis-Menten equation:

\[
v_0 = \frac{V_{max} [S]}{K_m + [S]},
\]

where \( V_{max} \) = maximum reaction velocity and \( K_m \) = Michaelis constant. The \( V_{max} \) is the product of \( k_{cat} \) and the total enzyme concentration, \([E]_0\):

\[
V_{max} = k_{cat} [E]_0.
\]

The \( K_m \) is given by the rate constants in equation 1:

\[
K_m = \frac{k_f + k_{cat}}{k_f}.
\]

For each substrate concentration, the assay data were converted from FU to micromolar by subtraction of the baseline and division by the slope (FU/μM) measured by 5-FAM titration. The initial reaction velocities \( v_0 \) were calculated by linear regression analysis on the data from the first 10 min and then fitted to the Michaelis-Menten model (equation 2) by nonlinear regression analysis with the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). The catalytic rate constant was calculated using equation 3.

RESULTS

Solubility and Quenching Efficiency

The McIlvaine’s buffer system was chosen for our study because its wide buffering range (pH 3–8) enabled an investigation of the pH dependence of the enzyme activity. However, protonation of 5-FAM at lower pH values disrupted the signal. Therefore, the pH range used was between 5.50 and 6.65. Brij35 was added to a final concentration of 0.01% (wt/vol) to prevent nonspecific binding to the well surface. The solubility of the substrate was in the range of a few micromolar without the addition of DMSO. The inclusion of 10% (vol/vol) DMSO in the assay buffer increased the solubility of the substrate to 30, 40, and 50 μM at pH 6.65, 6.00, and 5.50, respectively. The quenching efficiency of the substrate was calculated to be 95 ± 1%.

Analysis of the Data by Michaelis-Menten Kinetics

The usefulness of the assay is demonstrated in Figure 2, which shows the emission signal of the substrate incubated with unglycosylated bovine chymosin at pH 6.00. The initial velocities for the first 10 min are plotted in Figure 3 and the kinetic data as the Lineweaver-Burk Plots are shown in Figure 4. At pH 6.65, the low solubility of the substrate gives uncertainty to the initial velocities at higher substrate concentration and the corresponding Lineweaver-Burk plot (Figure 4). The initial velocities are comparable at pH 5.50 and 6.00, but an order of magnitude lower at pH 6.65.

The Michaelis-Menten kinetic fits to the experimental data are illustrated in Figure 3 and the corresponding parameters listed in Table 2. Overall, the \( K_m \) value increases and the \( k_{cat} \) value decreases with increasing pH with the largest relative change from pH 6.00 to 6.65 (e.g., \( K_m \) = 26 ± 2 and 38 ± 11 μM and \( k_{cat} \) = 28 ± 1 and 8 ± 2 s⁻¹ for unglycosylated bovine chymosin at pH 6 and 6.65, respectively). Camel chymosin has a lower \( K_m \) and a higher \( k_{cat} \) than bovine chymosin at all 3 pH values. The doubly glycosylated camel chymosin has a higher \( k_{cat} \) than the unglycosylated enzyme at pH
5.50 (59 ± 3 and 47 ± 4 s\(^{-1}\)) and 6.00 (65 ± 3 and 53 ± 3 s\(^{-1}\)).

For both enzymes the catalytic efficiency, \(k_{\text{cat}}/K_m\), decreased significantly with increasing pH; the catalytic efficiency of camel chymosin decreased roughly 10-fold from pH 6.00 to 6.65 (5.0 ± 0.8 and 0.6 ± 0.2 \(\mu M^{-1}s^{-1}\) for the unglycosylated variant). Camel chymosin has a higher catalytic efficiency than bovine chymosin (e.g., 6.8 ± 1.6 compared with 1.3 ± 0.1 \(\mu M^{-1}s^{-1}\) for the unglycosylated variants at pH 5.50); glycosylation does not affect the catalytic efficiency significantly.

**DISCUSSION**

With the traditional REMCAT method, the milk-clotting activities of camel and bovine chymosin were

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**Figure 2.** Illustration of the fluorescence intensity measured with the assay. For viewing clarity, only the data for the unglycosylated bovine chymosin (Bov) at pH 6.00 are shown and only every second data point is plotted. The symbols show the mean value and SEM of 3 measurements at the indicated substrate concentration with a fixed enzyme concentration of 50 \(pM\). (a) The full substrate concentration range; (b) a more detailed view of the data in the lower substrate concentration range; FU = fluorescence units.
determined to be 462 international milk-clotting units (IMCU) per milligram and 223 IMCU/mg, respectively, resulting in a 2-times-higher milk-clotting activity of camel chymosin (Langholm Jensen et al., 2013). In comparison, from the assay in our study (using the short substrate) the catalytic efficiencies were 0.6 ± 0.2 and 0.20 ± 0.07 μM⁻¹S⁻¹ at pH 6.65 for unglycosylated camel and bovine chymosin, respectively. The values for the glycosylated enzymes were similar, at 0.68 ± 0.23 and 0.18 ± 0.09 μM⁻¹S⁻¹ for glycosylated camel and bovine chymosin, respectively (Table 2). These results show 2 to 3 times higher catalytic efficiency overall for
camel chymosin and that it is the enzymatic step of milk-clotting that determines the differences between milk clotting by bovine and camel chymosin. This result suggests that the aggregation step is independent of the choice of chymosin.

A decrease in pH is accompanied by an increase in the catalytic efficiency of both bovine and camel chymosin, which is reflected in the increase of the catalytic rate constant and the decrease in the Michaelis-Menten constant. The optimum pH of bovine chymosin was determined to be 3.5 by Foltmann (1969), who explained that the catalytic rate increases as the pH approaches this value. At pH 5.50, the \( k_{cat} \) value of bovine chymosin compares well with the value obtained previously for unlabeled bovine \( \kappa-CN \) 98–112 [52.7 ± 3 s\(^{-1} \)] from Visser et al. (1987) compared with 28 ± 1 s\(^{-1} \)]. However, at pH 6.65, the \( k_{cat} \) value is lower than seen previously at pH 6.6 [49.3 ± 1.4 s\(^{-1} \)] from Visser et al. (1987) compared with 14 ± 2 s\(^{-1} \)]. A similar variation in catalytic rate was found for camel chymosin, as expected, because the 2 enzymes possess similar catalytic machinery (Langholm Jensen et al., 2013). At pH 5.50, 6.00, and 6.65, camel chymosin has significantly higher \( k_{cat} \) values than bovine chymosin. The doubly glycosylated variant of camel chymosin has a slightly higher \( k_{cat} \) value than the unglycosylated variant.

The addition of the fluorescent labels to the substrate, including the replacement of Pro110 with the more flexible Lys, may have little or no effect on substrate binding, as suggested by similar \( K_m \) values. The Michaelis-Menten constant at pH 6.65 for bovine chymosin is of the same order of magnitude as the one determined by Visser et al. (1987) for unlabeled bovine \( \kappa-CN \) 98–112 (29 ± 2 \( \mu M \)) at pH 6.6 and 30°C, compared with 38 ± 11 \( \mu M \) in our study). Previous studies showed a decrease in milk-clotting activity of doubly glycosylated camel chymosin due to the proximity of the glycosylation to the binding cleft (Langholm Jensen et al., 2013). As expected, glycosylation does not have any significant effect on binding of the short peptide substrate. It seems likely that the increase in substrate affinity from pH 6.65 to 6.00 is due to the increased positive charge of the His-Pro cluster of bovine \( \kappa-CN \) (Gustchina et al., 1996) that interacts with negatively charged residues in bovine chymosin as suggested by Gilliland et al. (1990) and Newman et al. (1991). The increased substrate affinity of camel chymosin can be related to differences in charged and hydrophobic residues as well as a more open binding cleft and a mobile N terminus, which has been described in depth previously (Langholm Jensen et al., 2013).

The high quantum yield of fluorescein results in an excellent emission signal even at low substrate concentrations, but its short Stokes shift causes significant self-quenching (Lakowicz, 2006) even at concentrations below 1 \( \mu M \), which requires data correction. It would be desirable to test chymosin and other coagulants over a broader pH range, but this is not possible due to the protonation of fluorescein as pH is decreased. A possible solution is the use of the Edans-Dabcyl pair, which has been used in other proteolytic assays (Matayoshi et al., 1990, Holskin et al., 1995, Grahn et al., 1998) and may lead to an improvement of the assay.

The assay can be used under milk clotting-like conditions (Fox and McSweeney, 1998) at low substrate concentrations for screening purposes. For determination of the Michaelis-Menten parameters, it was necessary to add DMSO to a final concentration of 10% (vol/vol) to increase solubility of the substrate. A study on a different peptidases showed that addition of up to 10% DMSO did not affect the enzymatic reaction (Grum-Tokars et al., 2008). To obtain a more precise \( K_m \) value it is necessary to increase the solubility of the substrate. One approach is to include Arg97 and 113-Asn-Gly-Asp-Lys-Thr-Glu-118 of \( \kappa-CN \) to improve solubility, though this might extend the enzyme-substrate interactions beyond the binding cleft.

One challenge during cheese manufacture is the inactivation of remaining chymosin in the whey to avoid further hydrolysis of the whey proteins (Hayes et al., 2002). The assay presented in our study would provide a quicker and more sensitive method for screening of

### Table 2. Michaelis-Menten kinetic parameters and SE for unglycosylated (Bov), single glycosylated (BovGlyc) bovine chymosin, unglycosylated (Cam), and doubly glycosylated (CamGlyc) camel chymosin

<table>
<thead>
<tr>
<th>Item(^1)</th>
<th>pH 6.65</th>
<th>pH 6.00</th>
<th>pH 5.50</th>
<th>pH 6.65</th>
<th>pH 6.00</th>
<th>pH 5.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_m (\mu M) )</td>
<td>38 ± 11</td>
<td>26 ± 2</td>
<td>22 ± 2</td>
<td>37 ± 16</td>
<td>28 ± 4</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>( k_{cat} (s^{-1}) )</td>
<td>8 ± 2</td>
<td>7 ± 2</td>
<td>28 ± 1</td>
<td>28 ± 4</td>
<td>27 ± 2</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>( k_{cat}/K_m (\mu M^{-1}s^{-1}) )</td>
<td>0.20 ± 0.07</td>
<td>0.18 ± 0.09</td>
<td>0.18 ± 0.08</td>
<td>0.11 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

\(^1\)\( K_m = \) Michaelis constant; \( k_{cat} = \) catalytic rate constant.
residual chymosin activity in the whey. For instance, using a 35-IMCU/L dosage of coagulant, corresponding to 4.4 nM bovine chymosin or 2.1 nM camel chymosin, the assay could readily detect a 1% residual activity at 0.5 μM substrate concentration and pH 6.65 with a limit of detection defined as 3 times the standard deviation of the signal of the blank (see Figure 2).

CONCLUSIONS

We have designed a FRET assay based on a substrate derived from bovine κ-CN. The assay is suitable for determination of Michaelis-Menten kinetics, for detection of milk-clotting activity, and can be carried out easier and faster than the traditional assay. Measurements of bovine and camel chymosin show that the enzymatic step of milk clotting is the determining step in the improved milk-clotting activity of camel chymosin.

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