ABSTRACT

The first step in cheesemaking is the milk clotting process, in which κ-caseinolytic enzymes contribute to micelle precipitation. The best enzyme for this purpose is chymosin because of its high degree of specificity toward κ-casein. Although recombinant bovine chymosin is the most frequently used chymosin in the industry, new sources of recombinant chymosin, such as goat, camel, or buffalo, are now available. The present work represents a comparative study of 4 different recombinant chymosins (goat and buffalo chymosins expressed in *Pichia pastoris*, and bovine and camel chymosin expressed in *Aspergillus niger*). Recombinant goat chymosin exhibited the best catalytic efficiency compared with the buffalo, bovine, or camel recombinant enzymes. Moreover, recombinant goat chymosin exhibited the best specific proteolytic activity, a wider pH range of action, and a lower glycosylation degree than the other 3 enzymes. In conclusion, we propose that recombinant goat chymosin represents a serious alternative to recombinant bovine chymosin for use in the cheesemaking industry.

Key words: recombinant chymosins, comparative analysis, κ-casein

Short Communication

Chymosin (EC 3.4.23.4) is an aspartic protease, mainly produced in the abomasum of suckling ruminants, that has a bilobulated structure with a deep groove containing the active site (Asp<sup>32</sup> and Asp<sup>215</sup>; Tang et al., 1978). Chymosin is routinely used in the cheesemaking industry to generate cheese curds. The enzyme hydrolyzes the linkage between Phe<sup>105</sup> and Met<sup>106</sup> in the κ-casein molecule, resulting in the destabilization of casein micelles and causing flocculation. The final product of this flocculation process is the generation of milk curds (Vasbinder et al., 2003).

Traditionally, chymosins were obtained from the abomasum of suckling calves, but this source has now become insufficient to supply the increasing worldwide demand. Hence, today the main source of chymosin is the genetically engineered bovine recombinant chymosin expressed in a variety of microorganisms (Kumar et al., 2010). However, recombinant chymosins are currently available from other ruminants, such as goat (Vega-Hernández et al., 2004), camel (Kappeler et al., 2006), and buffalo (Vallejo et al., 2008). The present work focuses on the comparative study of the 4 recombinant chymosins currently available.

Materials

Recombinant bovine chymosin (BtRC) and recombinant camel chymosin (CdRC) were Chymax and Chymax M from Chr. Hansen A/S (Hørsholm, Denmark). The BtRC and CdRC were dialyzed against 0.01 M potassium phosphate buffer, pH 6. Recombinant goat chymosin (ChRC) was expressed in *Pichia pastoris* for this study. Recombinant buffalo chymosin (BbRC) was produced according to Vallejo et al. (2008).

The microbial strains used in our study were *Escherichia coli* TOP10 and *P. pastoris* X33 (both from Invitrogen, Carlsbad, CA). Plasmid pQSec1, containing the chymosin gene from *Capra hircus*, was obtained from Félix Claverie-Martín (Molecular Biology Laboratory, Research Unit, Nuestra Señora de Candelaria University Hospital, Santa Cruz de Tenerife, Spain). Plasmid pGAPZα (Invitrogen) was used as the expression vector for *P. pastoris*. The strain *E. coli* TOP10 was cultured in Luria Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, and 10 g/L of NaCl) supplemented with kanamycin (50 μg/mL) or zeocin (25 μg/mL) for recombinant plasmid selection. *Pichia pastoris* X33 was grown in yeast extract peptone dextrose medium (10 g/L of yeast extract, 10 g/L of peptone, and 20 g/L of glucose) and supplemented with zeocin (100 g/mL) to select for recombinant yeast colonies.

Construction of Goat Pro-Chymosin Expression Vector (pGAPZα-CH)

The goat pro-chymosin 1,172-kbp DNA fragment was amplified by PCR from the plasmid pQSec1, using iProof High-Fidelity DNA Polymerase (Bio-Rad, Her-
cules, CA) and specific primers containing recognition sites for EcoRI (forward 5’-ggaattcgccttGCTGAGAT-TACCAGAATCC-3’ and reverse 5’-ggaattcgccttTCA-GATAGCTTTAGCCAGC-3’). The resulting product was digested with EcoRI (Takara, Japan) and ligated to the previously EcoRI-digested and dephosphorylated (using shrimp alkaline phosphatase, Takara) pGAPZα vector. The DNA construct thus obtained was used to transform E. coli TOP10 competent cells, and the correct orientation of the DNA was determined by KpnI (Takara) digestion.

**Transformation of P. pastoris**

Plasmid vectors containing the pro-chymosin DNA with the correct orientation were then linearized using the restriction enzyme BspHI (New England Biolabs, Ipswich, MA) and introduced into P. pastoris by electroporation, as described by Vallejo et al. (2008).

**BbRC and ChRC Production in P. pastoris**

The recombinant P. pastoris strains were cultured for 48 h in yeast extract peptone dextrose medium at 30°C in a rotary shaker at 300 rpm. One liter of culture was centrifuged at 5,000 × g for 15 min. The supernatant was then warmed to 37°C, adjusted with sodium sulfate (10.5%) and polyethyleneglycol 8000 (4%), and centrifuged at 5,000 × g for 15 min (Heinsohn et al., 1992). The upper phase was then removed, diluted with 3 volumes of water, and loaded onto a 20-mL DEAE-Biogel A (BioRad) column equilibrated with 50 mM l-histidine, pH 5.8. The column was washed with 14 volumes of the same buffer, and the recombinant chymosin was eluted with 100 mL of 50 mM l-histidine, pH 5.8, containing 2 M NaCl. Finally, the eluted enzyme was dialyzed against a 0.01 M potassium phosphate buffer, pH 6.

Protein concentration was assessed by the Bradford method (Bradford, 1976), the Coomassie Protein Assay Kit (Pierce Biotechnology, Rockford, IL), and by SDS-PAGE. Polyacrylamide gel electrophoresis was performed, according to Laemmli (1970), in a mini Protean-III cell (Bio-Rad) with 500 ng of each recombinant chymosin. The molecular weight of the proteins was determined using Precision Plus Protein Standard (Bio-Rad) and Quantity One software (Bio-Rad).

**Determination of Chymosin Activity and Kinetic Parameters**

The assay, as described by Ageitos et al. (2006), was carried out to test the κ-caseinolytic activity of the recombinant enzymes. Activity was expressed as a percentage of the maximum activity value.

Michaelis-Menten plots (Dixon et al., 1979) were performed by using increasing substrate concentrations, as described by Ageitos et al. (2006). The enzymes’ maximum rate (V max) and Michaelis constant (Km) values were calculated using the Lineweaver-Burk method (Dixon et al., 1979). The inhibitor constant (Ki) values were obtained by using increasing substrate concentrations in the presence of the inhibitor pepstatin at a concentration of 1.66 mM (BbRC, BtRC, and ChRC), and 16.66 mM (CdRC). All kinetic parameters were determined in triplicate.

**Determination of Temperature and pH Range of Activity**

For the determination of the temperature range of activity, the recombinant chymosins were incubated for 1 h at temperatures ranging from 10 to 70°C. Enzymatic activity was also tested for 1 h at different pH values (from 2.0 to 9.0). The buffers used for this purpose were 500 mM sodium citrate (pH 2–3.5), 500 mM sodium acetate (pH 4–5.5), 500 mM potassium phosphate (pH 6–7.5), and 500 mM Tris-HCl (pH 8–9). The enzymatic activity was then measured as described above.

**Determination of General Proteolytic Activity and Enzyme Thermostability**

General proteolytic activity was measured, as described by Twining (1984) and modified by Ageitos et al. (2006), using the fluorescein-labeled substrate thio-carbamoyl-casein (Sigma-Aldrich, St. Louis, MO). The results obtained were used to determine the specific proteolytic activity on κ-casein compared with the general proteolytic activity of the enzyme on other caseins.

The enzymes BbRC, BtRC, ChRC, and CdRC were incubated for 24 h at temperatures ranging from 5 to 70°C, with 5°C steps. The residual activity of the samples was then measured by the κ-caseinolytic assay, as described above.

**BbRC and ChRC Production in P. pastoris**

The goat pro-chymosin gene (1,101 bp) was cloned into the plasmid pGAPZα and expressed under the control of P. pastoris gap promoter, using the signal peptide from Saccharomyces cerevisiae α factor. Twenty-five transformants were selected after screening and analyzed for chymosin production. This allowed us to identify the highest chymosin-producing clone. Produt-
tion of BbRC and ChRC was carried out as described by Vallejo et al. (2008).

Recombinant Chymosin Profiles

As shown in Figure 1A, both BbRC and CdRC recombinant chymosins migrated in PAGE as 2 protein bands (with molecular weights of 39.1 and 36.2 kDa for BbRC, and 37.58 and 36.86 kDa for CdRC). This reflects the high glycosylation pattern of these 2 enzymes compared with BtRC and ChRC, this pattern being related to the enzymatic activity, as described by van den Brink et al. (2006). On the other hand, BtRC and ChRC (Figure 1A) appeared to be scarcely glycosylated (band at 35.8 kDa for BtRC and at 36.9 kDa for ChRC), because overloading with a considerable amount of protein was required to visualize the glycosylated form of these 2 chymosins (Figure 1B).

Temperature and pH Range of Activity

Camel chymosin showed the broadest range of temperature tolerance, exhibiting a residual activity of more than 80% (\(RA_{80}\)) at temperatures ranging from 25 to 45°C, with a maximum activity at 40°C (Figure 2A). This property could have some interesting applications in cheesemaking, either to catalyze milk clotting at low temperatures or to accelerate the process at 45°C. Buffalo chymosin displayed an RA\(_{80}\) at temperatures ranging from 30 to 40°C, with a maximum activity at 37°C (Figure 2A). On the other hand, both BtRC and ChRC showed an RA\(_{80}\) at temperatures ranging from 35 to 40°C, with maximum activity at 40 and 45°C, respectively (Figure 2A).

With respect to pH range, ChRC had the broadest range of action, because it maintained an RA\(_{80}\) at pH values between 3 and 6.5 (Figure 2B), whereas CdRC had the narrowest pH range for RA\(_{80}\) (5.5 to 6.5). Bovine and buffalo chymosins displayed a similar pH range of action with values between those obtained for ChRC and CdRC (Figure 2B).

Kinetic Parameters

The ChRC displayed the best catalytic efficiency; it was 6, 11, and 16 times more efficient than BbRC, BtRC, and CdRC, respectively (Table 1). As shown in Table 1, the catalytic efficiency of the enzymes was strongly related to the amount of \(\kappa\)-casein of the different milks. The high values of catalytic efficiency found for ChRC can be explained in terms of the higher percentage of \(\kappa\)-casein present in the goat milk (20.4 %; Park et al., 2007) compared with 15.4% (Zicarelli, 2004), 13% (Eigel et al., 1984), and 3.5% (Kappeler et al., 1998) for the buffalo, cow, and camel milks, respectively.

The \(K_m\) values obtained for the enzymes indicated that ChRC had higher substrate affinity than the...
other recombinant chymosins (Table 1), whereas BtRC showed the lowest affinity (Table 1); this is despite the fact that the substrate used in this analysis was bovine κ-casein. The lowest catalytic turnover value was determined for CdRC, which explained the low catalytic efficiency shown by this enzyme. As seen in Table 1, the affinity and turnover values for ChRC were well balanced, and resulted in its higher catalytic efficiency compared with the other recombinant chymosins (Table 1).

The inhibition studies revealed 3 different behavior patterns (Figure 3). The enzymatic activity of ChRC was the most affected by pepstatin inhibition, followed by BbRC and BtRC, with CdRC being the least sensitive to the inhibitor. These data strongly suggest that differences exist in the active sites of these 4 recombinant chymosins.

### Enzymatic Activity and Thermostability

The κ-caseinolytic activity per nanogram of chymosin was determined using bovine chymosin as reference (100% of activity). As shown in Table 2, ChRC had a 50% higher activity than that of BbRC, BtRC, and CdRC. In the case of BbRC, the value was lower than expected for its catalytic efficiency, which could be due to the higher degree of glycosylation of this enzyme (van den Brink et al., 2006). On the other hand, CdRC showed a greater κ-caseinolytic activity than that expected for its catalytic efficiency; this discrepancy was also observed by Kappeler et al. (2006).

To determine the substrate specificity of the different chymosins, we compared their κ-caseinolytic activity (Ageitos et al., 2006) with their caseinolytic activity (Twining, 1984). As shown in Table 2, ChRC displayed the best ratio, followed by BbRC and BtRC, with CdRC having the worst ratio. This finding may be

### Table 1. Determination of enzymatic-kinetic constants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BbRC</th>
<th>BtRC</th>
<th>CdRC</th>
<th>ChRC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (mM)</td>
<td>7.59E−04 ± 4.26E−05</td>
<td>8.19E−03 ± 2.68E−04</td>
<td>5.97E−04 ± 3.59E−05</td>
<td>3.89E−04 ± 4.29E−05</td>
</tr>
<tr>
<td>$K_{cat}$ (s−1)</td>
<td>1.04E−03 ± 1.13E−04</td>
<td>6.21E−03 ± 2.78E−04</td>
<td>3.19E−04 ± 1.92E−05</td>
<td>3.31E−03 ± 2.54E−04</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>1.70 ± 0.06</td>
<td>1.63 ± 0.11</td>
<td>16.44 ± 1.03</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>Catalytic efficiency (mM−1 s−1)</td>
<td>1.37</td>
<td>0.76</td>
<td>0.53</td>
<td>8.49</td>
</tr>
<tr>
<td>κ-Casein (% vs. total caseins)</td>
<td>15.4±</td>
<td>13±</td>
<td>3.5±</td>
<td>20.4±</td>
</tr>
</tbody>
</table>

1BbRC = recombinant buffalo chymosin; BtRC = recombinant bovine chymosin; CdRC = recombinant camel chymosin; ChRC = recombinant goat chymosin.

2$K_m$ = Michaelis constant; $K_{cat}$ = turnover rate; $K_i$ = inhibitor constant.


5Kappeler et al. (1998).

6Park et al. (2007).

### Table 2. Specific enzymatic activity and relationship between κ-casein activity and general proteolytic activity

<table>
<thead>
<tr>
<th>Chymosin</th>
<th>Specific enzymatic activity</th>
<th>κ-Casein vs. general proteolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>BbRC</td>
<td>105.22 ± 5.22</td>
<td>6.78</td>
</tr>
<tr>
<td>BtRC</td>
<td>100.00 ± 4.89</td>
<td>6.10</td>
</tr>
<tr>
<td>CdRC</td>
<td>98.56 ± 3.86</td>
<td>5.48</td>
</tr>
<tr>
<td>ChRC</td>
<td>148.26 ± 5.59</td>
<td>8.78</td>
</tr>
</tbody>
</table>

1BbRC = recombinant buffalo chymosin; BtRC = recombinant bovine chymosin; CdRC = recombinant camel chymosin; ChRC = recombinant goat chymosin.

2Specific enzymatic activity per nanogram of chymosin, expressed as percentage of bovine chymosin.

3Enzymatic activity determined using κ-casein as substrate, as described by Ageitos et al. (2006) compared with that using all milk caseins as substrate, as described by Twining (1984).
explained in terms of the different milk composition for the 4 mammals. In fact, camel milk is not clotted by bovine chymosin, whereas cow milk is clotted by camel chymosin (Kappeler et al., 2006).

As shown in Figure 4, thermostability of the 4 recombinant chymosins did not differ when the enzymes were incubated at temperatures between 5 and 25°C. However, at temperatures between 25 and 35°C, CdRC showed greater stability than the other 3 enzymes. The different geographical distribution of these species may reflect the tolerance of the respective chymosins to temperature variations.

According to the data herein, we conclude that the goat chymosin is the most suitable recombinant chymosin for the requirements of the cheesemaking industry, because it is scarcely glycosylated, shows a high residual activity at different pH values, and displays the best catalytic efficiency and κ-casein specificity of the 4 chymosins analyzed.

ACKNOWLEDGMENTS

We thank Felix Claverie Martin for the Plasmid pQ-Sec1 and Hans van den Brink for the gift of Chymax M. The authors express their gratitude to A. Sánchez (Sydney University, Australia) for critically reading the manuscript.

REFERENCES


