ABSTRACT

The bovine endopeptidase cathepsin D was investigated regarding its temperature-dependent inactivation and ability to form bitter peptides within a spiked model fresh cheese. Cathepsin D was found to be more susceptible than other milk endogenous peptidases to temperature treatments in skim milk. Inactivation kinetics revealed decimal reduction times of 5.6 min to 10 s in a temperature range from 60 to 80°C. High temperature and ultra-high temperature (UHT) treatments from 90 to 140°C completely inactivated cathepsin D within 5 s. A residual cathepsin D activity of around 20% was detected under pasteurization conditions (72°C for 20 s). Therefore, investigations were done to estimate the effect of residual cathepsin D activity on taste in a model fresh cheese. The UHT-treated skim milk was spiked with cathepsin D and acidified with glucono-δ-lactone to produce a model fresh cheese. A trained bitter-sensitive panel was not able to distinguish cathepsin D–spiked model fresh cheeses from the control model fresh cheeses in a triangle test. Model fresh cheese samples were also analyzed for known bitter peptides derived from casein fractions using a HPLC–tandem mass spectrometry (MS) approach. In accordance with the sensory evaluation, the MS analyses revealed that the bitter peptides investigated within the cathepsin D–spiked model fresh cheese were not found or were below the limit of detection. Even though cathepsin D may be present during the fermentation of pasteurized milk, it does not seem to be responsible for bitter peptide formation from milk proteins on its own. Key words: cathepsin D, fresh cheese, bitter peptides, heat inactivation

INTRODUCTION

Bovine milk is an important resource for human consumption. It contains valuable proteins and micronutrients that contribute to its high nutritional value (Fox et al., 2015). It also inherits a broad range of endogenous enzymes, such as lipases, oxidases, glycosidases, and peptidases (Fox and Kelly, 2006a, b). Exogenous enzymes may be present in the milk during the storage and processing of raw milk, due either to secretion of proteolytic enzymes by psychrophilic spoilage bacteria during storage at low temperature or through the metabolism of starter cultures (Hantsis-Zacharov and Halpern, 2007; Liu et al., 2010). For example, Pseudomonas spp. can secrete peptidases into raw milk at low temperature. These peptidases are resistant to later UHT treatment and can cause destabilization during storage (Baur et al., 2015). Other negative effects of proteolytic activities can be lower cheese yields (Franceschi et al., 2020) and the formation of bitter peptides (Lemieux and Simard, 1992; Baum et al., 2013). It is important to eliminate any undesirable proteolytic enzyme activities before or during production to maintain the quality of dairy products. In most cases, this is accomplished by appropriate heat treatments. Some of the peptidases discussed are known to overcome thermization or even UHT treatments (Baur et al., 2015; Deeth, 2021; Graf et al., 2021; Kelly and Larsen, 2021; Stoeckel et al., 2016). The most extensively studied endogenous peptidase plasmin (EC 3.4.21.7) is active at neutral pH (Fox et al., 2015). It not only exhibits high stability at high temperatures but is also activated at time-temperature combinations ranging from 72°C for 180 s to 85°C for 30 s (Stoeckel et al., 2016). The production of many dairy products is accomplished by lowering the pH; thus, the activities of neutral peptidases decrease, and the activities of aspartic peptidases are switched on. The main aspartic endopeptidase in bovine milk is cathepsin D (EC 3.4.23.5), which is associated with the count of immune
cells within the milk (Larsen et al., 2004; Moradi et al., 2021). Cathepsin D is able to survive certain heat treatments (Table 1) and can hydrolyze all casein fractions present in bovine milk (McSweeney et al., 1995; Hurley et al., 2000). A possible negative influence of cathepsin D on dairy products was discussed by several authors (Kaminogawa and Yamauchi, 1972; Hayes et al., 2001). However, to date, the temperature dependency of cathepsin D activity, especially in milk matrices, has not been systematically investigated.

All published articles report that residual cathepsin D activity was determined after the heating step. Cathepsin D activity might cause problems especially in acidified milk products, as it remains active during production and storage. Regarding experiments in milk, Hayes et al. (2001) postulated a protective effect of the milk proteins. The effect of cathepsin D has rarely been investigated in dairy products. Hurley et al. (2000) detected milk protein hydrolysis in quark using reverse-phase HPLC and ascribed it to the cathepsin D activity remaining in pasteurized skim milk (74°C for 15 s). Similarly, changes in milk protein patterns were observed during the storage of feta cheese that was produced from pasteurized (72°C for 15 s) and ultrafiltered milk (Larsen et al., 2000). Several quality defects in the final products are possible due to residual endopeptidase activity. In the case of cathepsin D, which preferentially cleaves between hydrophobic amino acids (Handley et al., 2001), bitterness can emerge due to the hydrophobic peptides formed (FitzGerald and O’Cuinn, 2006; Ewert et al., 2019; Kelly and Larsen, 2021). Some studies reported bitterness in dairy products such as Gouda and concentrated fresh cheese. After analysis of the bitter peptide sequences, some cleavage sites were traced back to cathepsin D (Toelstede and Hofmann, 2008; Sebald et al., 2018).

However, no data are available to bring together the stability of cathepsin D during heat treatments, its activity during storage, and a possible deriving influence on the taste impression of the final product. Therefore, in this study, we investigated the temperature dependency of cathepsin D during heating in skim milk over a broad temperature range. Furthermore, model fresh cheeses were produced and spiked with active cathepsin D to evaluate the emerging bitterness using a sensory panel. Additionally, the model fresh cheeses were analyzed via HPLC–tandem mass spectrometry (MS/MS) to discover the presence of the known bitter peptides reported by Sebald et al. (2018).

Table 1. Literature regarding the temperature dependency of bovine cathepsin D

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Temperature and time</th>
<th>Residual cathepsin D activity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate buffer (pH 4)</td>
<td>50°C</td>
<td>~98</td>
<td>Kaminogawa and Yamauchi (1972)</td>
</tr>
<tr>
<td></td>
<td>60°C, 10 min</td>
<td>~70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70°C, 10 min</td>
<td>Inactivated</td>
<td></td>
</tr>
<tr>
<td>Skim milk2</td>
<td>72°C, 15 s</td>
<td>8</td>
<td>Hayes et al. (2001)</td>
</tr>
<tr>
<td>Skim milk3</td>
<td>72°C, 15 s</td>
<td>~50</td>
<td>Larsen et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>85°C, 15 s</td>
<td>~17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99°C, 15 s</td>
<td>~9</td>
<td></td>
</tr>
</tbody>
</table>

1Activity determined using hemoglobin.
2Activity determined using Pro-Thr-Glu-Phe-[NO2-Phe]-Arg-Leu and reversed-phase HPLC.
3Activity determined in milk serum using hemoglobin; values are estimated from Figure 5 in Larsen et al. (2000).

**MATERIALS AND METHODS**

**Chemicals and Material**

The chemicals used were of analytical grade and purchased from Merck KGaA, Carl Roth GmbH, and AppliChem. Bovine cathepsin D (isolated from bovine spleen) was purchased from Merck KGaA. Low-fat skim milk (0.3% wt/wt fat; Gut & Günstig) was used to determine the thermal inactivation of cathepsin D and preparation of cathepsin D–spiked model fresh cheese.

**Determination of Cathepsin D Activity**

Cathepsin D activity was determined by use of the fluorescence substrate MocAc-GKPILFFRLK(Dnp) R-NH₂ (PeptaNova) developed by Yasuda et al. (1999). The assay was conducted kinetically at 37°C in a volume of 100 µL, consisting of 80 µL of sodium acetate buffer (pH 3.6), 10 µL of a 100 µM substrate solution (dissolved in dimethyl sulfoxide), and 10 µL of cathepsin D solution. If necessary, the volume of cathepsin D solution was varied while the buffer volume was adjusted accordingly. All activity and blank assays were done in triplicate. The fluorescence was determined by exciting the sample at 355 nm and measuring the signal emitted at 460 nm in a fluorescence spectrometer (Fluoroskan Ascent FL, Labsystems, Fisher Scientific GmbH).
Investigation of the pH Effect on Cathepsin D Activity

The effect of pH on cathepsin D activity was determined in 2 different buffer systems. The pH was varied between 3.4 and 7 and the following buffers were used: 100 mM sodium acetate (pH 3.4–5.5) and 100 mM Bis-Tris (pH 5.5–7.0). A calibration curve was prepared for every pH value as the fluorescence of the substrate is affected by pH.

Investigation of the Heat Inactivation of Cathepsin D

The thermal stability of cathepsin D was determined in UHT skim milk. The heating step was performed in a PCR thermocycler (Jena Analytics). A volume of 95 µL of skim milk was added to a 200-µL PCR reaction tube and mixed with 5 µL of a 1 mg mL−1 cathepsin D solution. After spinning down (10 seconds, 2,860 × g, room temperature), the respective sample was heated to temperatures ranging from 60 to 95°C for time spans between 5 s and 5 min. The thermocycler program started at 5°C, heated to the selected temperature at 6°C s−1 and cooled down to 5°C at 3°C s−1. Afterward, the samples were stored on ice until preparation for determination of cathepsin D activity.

Additionally, the stability of cathepsin D to UHT treatment was investigated. As the thermocycler cannot cover temperatures >99°C, the UHT treatment was performed in a batch heating system described by Dogan et al. (2009). A volume of 1.5 mL of UHT-treated skim milk was spiked with cathepsin D and added to a stainless-steel tube. This tube was hooked on the cover plate of an isolated vessel that was instantly heated to 140°C with hot steam under pressure, and the temperature was held for 10 s. Subsequently, the sample was cooled with ice water to <10°C. The temperature progress was followed within a reference tube. After the heat treatment, the samples were stored on ice until preparation for determination of cathepsin D activity.

Extraction of Cathepsin D from Skim Milk Samples

The sample preparation for cathepsin D activity determination was done according to Larsen et al. (1993) with modifications. The sample was acidified to a pH of 3.5 using 1 mol L−1 sulfuric acid, yielding a final concentration of 20 mmol L−1 sulfuric acid within the sample. The disintegrated and precipitated caseins were separated via centrifugation (17,949 × g, 4°C, 10 min). The whey protein-containing supernatant was used for the cathepsin D activity assay.

Calculation of Inactivation Rate Constant, Half-Life, and Decimal Reduction Time

The first-order inactivation rate constant ($K_d$) was determined by use of equations [1] and [2] (Sadana, 1988; Baur et al., 2015):

$$\frac{E_t}{E_0} = e^{-K_d t}$$

converted to

$$\ln\left(\frac{E_t}{E_0}\right) = -K_d t,$$

where $E_t$ is the cathepsin D activity (pkat mL−1) after a heat treatment at a given temperature for the heating time $t$ in seconds, $E_0$ is the activity within an unheated sample, and $K_d$ is the inactivation rate constant in s−1.

In a plot of $\ln (E_t/E_0)$ over time at the respective temperature, $K_d$ corresponds to the slope of the regression line and is further used for the calculation of the half-life ($T_{1/2}$; equation [3]), and the decimal reduction time ($D_{10}$; equation [4]); $D_{10}$ is defined as the time necessary for a reduction to 1/10 at a certain temperature $\vartheta$ (Deeth, 2021):

$$T_{1/2} = \frac{\ln(2)}{K_d} \text{ and}$$

$$D_{10} = D \frac{\ln(10)}{K_d}.$$ 

Equation [5] was used to calculate the time needed to reduce cathepsin D to 1% of the initial activity:

$$T_{1/100} = \frac{\ln(100)}{K_d}.$$ 

Production of Cathepsin D–Spiked Model Fresh Cheese

The cathepsin D preparation was dissolved in sterile water, desalted in sterile water by use of PD-10 columns, and then sterile-filtered. Two types of model fresh cheese were prepared, adding 5 and 10 pkat mL−1 milk,
respectively. The activities were chosen based on the
general cathepsin D concentration of 0.4 µg mL\textsuperscript{-1}
milk\textsuperscript{-1} according to Larsen et al. (1996). This corresponded
to an estimated volumetric activity of 5 pkat mL\textsuperscript{-1}
milk\textsuperscript{-1} based on the activity and protein content of the
cathepsin D preparation used. A concentration of 1.1%
(wt/vol) glucono-δ-lactone (GDL, Alfa Aesar, Thermo
Fisher Scientific) was dissolved in UHT skim milk at room
temperature, followed by the addition of sterile
cathepsin D. The preparations were divided into batch-
es of 200 mL, which were filled into autoclaved 25-mL
plastic bottles, for a simulated fermentation. The latter
was conducted in a water bath at 23°C. After 19 h, the
bottles were centrifuged at 2,625 × \textit{g} at 20°C for 5 min
to remove the whey. The protein content of the model
fresh cheese fraction was determined using the bicin-
chonic acid assay using BSA (Serva Electrophoresis
GmbH) as a reference protein. The residual cathepsin D
activity within the whey fraction was determined as
-described before. The bottles were stored at 4°C until the
sensory evaluation. The experiment was done twice
on separate days. Afterward the samples were stored at
−20°C until further MS analysis (see below).

**Evaluation of the Microbial Contamination of the Model Fresh Cheeses**

The samples that were evaluated by a sensory panel
needed to be checked for microbial contamination to
guarantee the safety of the sensory panel members. The
samples were only used for sensorial analysis if they
were found to be completely devoid of any microbial
contamination. Therefore, no IRB approval was neces-
sary. Parts of these samples were diluted and incubated
on different agar plates. *Enterobacteriaceae* were de-
tected on violet red bile dextrose agar according to DIN
EN ISO 4833-1/A1 (DIN, 2020) after incubation for 24 h at 37°C. The total bacterial count was detected
as described by VDLUFA (2011) by using plate count
agar after 18 h of incubation at 30°C (both obtained
from Carl Roth GmbH + Co. KG). Additionally, Mac-
Conkey agar (Oxoid Ltd.) was used to detect coliform
bacteria after 18 h of incubation at 37°C.

**Sensorial Evaluation of Cathepsin D–Spiked Model Fresh Cheese**

Sensory panelists were selected by their ability to
sense the bitterness of a caffeine (Carl Roth GmbH)
dilution series (0.03 to 0.23 g L\textsuperscript{-1}; DIN 10959; DIN
2005). Then, the panelists were trained to taste 2 types
of fresh cheeses: one commercial fresh cheese labeled
“reference 1” (“Cremiger Quark mit Joghurt verfein-
ert,” Berchtesgardener Land Chiemgau eG) and one
model fresh cheese without cathepsin D labeled “refer-
ce 2.” Panelists were then asked to describe which
sample was perceived as more sour and which was more
bitter. Afterward, 2 triangle tests with the cathepsin
D–spiked model fresh cheese samples and reference 1
were conducted. Cheese samples were placed in plastic
cups and labeled with 3 random numbers. Two cups of
each set held the control model fresh cheese (reference
1) and 1 cup held the cathepsin D–spiked model fresh
cheese (5 or 10 pkat mL\textsuperscript{-1}, respectively). The task for
the panel was to detect the odd sample in each set.
Additionally, the panelists were asked to describe the
difference between the samples. The triangle test was
done twice on separate days. The trials were evaluated
regarding their level of significance, according to Law-
less and Heymann (2016).

**Quantification of Bitter Peptides in Cathepsin D–Spiked Model Fresh Cheese**

Sample preparation for the determination of bitter
peptides within the model fresh cheeses was conducted
according to Sebald et al. (2018). Water (HPLC-grade;
VWR International GmbH) was used for every step. One sample (1 ± 0.01 g) was extracted twice with 5
mL of 0.1% (vol/vol) formic acid (VWR International
GmbH) using 3 ± 0.1 g of glass beads. The sample
was centrifuged (13,806 × \textit{g}, 4°C, 10 min) after mix-
ing 3 times for 20 s each on a vortex. The internal
standard angiotensin I (1 g mL\textsuperscript{-1}, Bachem Holding AG)
was added to the sample, which was filtered (0.45 µm)
and stored at −20°C until analysis. The quantification
of the peptides extracted was conducted via liquid
chromatography-tandem MS by the Core Facility of the
University of Hohenheim (Stuttgart, Germany). The
peptides were separated on a C-18 column (Acquity
BEH C18 150 × 2.1 mm 1.7 µm, 130 Å, Waters Corp.)
coupled to an AB Sciex QTrap 5500 (Danaher Corp.).
Settings were chosen according to Sebald et al. (2018).
Calibration curves were prepared from the 17 standard
peptides (Peptides & Elephants). The peptides were
extracted from both fermentation experiments and
analyzed via HPLC-MS. For peptide analysis, Scaffold
5.0.1 (Proteome Software Inc.) was used, and for data
analysis and calculations, Microsoft Excel (Microsoft
Corp.) was used.

**RESULTS AND DISCUSSION**

**Optimized Sample Preparation for Cathepsin D Activity Determination**

The method described by Larsen et al. (1993) was
modified and used for the extraction of cathepsin D
from milk samples to facilitate the investigation of the temperature-dependent inactivation of cathepsin D. First, the casein micelles must be precipitated from the sample to allow an activity measurement via fluorescence signal. Larsen et al. (1993) used 40 mmol L⁻¹ sulfuric acid to acidify skim milk to pH 3.5 for the separation of casein micelles from the milk serum. A recovery experiment was done to determine the optimal concentration of sulfuric acid for precipitation of the casein micelles without co-precipitation of cathepsin D (Table 2). The samples remained cloudy at H₂SO₄ concentrations below 20 mmol L⁻¹ and resulted in an overestimation of the spiked cathepsin D activity (129% with 10 mmol L⁻¹ H₂SO₄). However, H₂SO₄ concentrations >30 mmol L⁻¹ presumably caused co-precipitation of cathepsin D and, therefore, resulted in low recovery (<53%). Thus, a concentration of 20 mmol L⁻¹ was used for sample preparation (103% recovery).

**pH Dependency of Cathepsin D**

The native pH of milk (~6.8) decreases to <5 during the fermentation of fresh cheese, while casein micelles aggregate and build up a 3-dimensional gel network (Walstra, 1990). This aggregated and concentrated casein then forms the final fresh cheese product after the removal of whey. Cathepsin D activity was first investigated in a buffer system to estimate the effect of the pH (see Figure 1). Maximal activity was determined at a pH of 4.5 in sodium acetate buffer (100% = 1,195 ± 52 pkat mL⁻¹). No activity was determined at a pH of 7. Although a buffer system is not comparable to milk, these findings can be used as a rough estimation of cathepsin D activity during the cheese fermentation. However, a protective effect of milk proteins during thermal treatment of cathepsin D is possible (Hayes et al., 2001); thus, cathepsin D might exhibit an even higher activity in cheese than determined in the buffer system. The pH decreases from 6.8 to 5.7 within the first 2 h of the simulated fermentation due to the dissociation of the added GDL (Lucey et al., 1998), while cathepsin D activity will increase (Figure 1). The pH further decreases to 4.7 after 18 to 19 h of fermentation. Thus, we can postulate that cathepsin D will find an optimum pH range to be active for most of the fermentation time.

**Inactivation Kinetics of Cathepsin D in Skim Milk**

Inactivation kinetics were determined by adding known cathepsin D activities to UHT skim milk. This “spiked milk” was exposed to a range of time-temperature combinations (60 to 95°C for 5 s to 5 min; see Figure 2). Raw milk can be thermized to low temperatures (57 to 68°C for up to 30 s) before further processing (German Milk Production Act, 2000). These conditions were not sufficient to inactivate cathepsin D completely. The residual cathepsin D activity was around 10% even after 5 min at 60°C. An increase in temperature to 65°C resulted in faster inactivation of cathepsin D, and 20% activity remained after 32 s of heating. Pasteurization at 72 to 75°C for 15 to 30 s is used for milk that is sold as “fresh milk” (German Milk Production Act, 2000; van der Over and Mayer, 2021). However, a residual cathepsin D activity of about 20% was determined after applying such pasteurization conditions (72°C for 20 s). When the temperature was increased to 75°C for 20 s, a residual cathepsin D activity of about 8% was measured. This inactivation behavior is similar to that in the study of Hayes et al. (2001), where cathepsin D was isolated from skim milk. There, a residual cathepsin D activity of 8% was estimated from a double exponential model at 72°C and 15 s. However, it was not clear whether isoforms of cathepsin D with different temperature dependencies were investigated (Deeth, 2021).

### Table 2. Spiked cathepsin D recovery from skim milk samples at different concentrations of sulfuric acid (100% activity was 1,061 ± 4 pkat mL⁻¹); means ± SD of 3 independent measurements are presented

<table>
<thead>
<tr>
<th>H₂SO₄ (mmol L⁻¹)</th>
<th>Cathepsin D activity (pkat mL⁻¹)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.369 ± 69</td>
<td>129.0 ± 5.0³</td>
</tr>
<tr>
<td>20</td>
<td>1.091 ± 14</td>
<td>103.0 ± 1.3</td>
</tr>
<tr>
<td>30</td>
<td>1.052 ± 12</td>
<td>99.0 ± 1.1</td>
</tr>
<tr>
<td>40</td>
<td>562 ± 30</td>
<td>53.0 ± 5.3</td>
</tr>
<tr>
<td>50</td>
<td>&lt;LOD²</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

³Overestimation of the measured signal due to incomplete milk protein precipitation.

²Limit of detection (0.02 pkat mL⁻¹).
cathepsin D activity in casein micelles and milk serum of about 60% after pasteurization treatment (72°C for 15 s). Surprisingly, a prolonged heating time of 60 s did not inactivate cathepsin D further.

After common pasteurization or high-temperature pasteurization (85 to 127°C), milk is used for the production of dairy products (German Milk Production Act, 2000). Additionally, these conditions are used to produce milk with an extended shelf-life (>10 d; van der Oever and Mayer, 2021). A strong decrease of cathepsin D activity was already determined after 10 s at 80°C (~6% residual activity). At higher temperatures of 90 or 95°C, no cathepsin D activity was determined after 5 s, even after prolonged assay incubation times. Again, this is in contrast to Larsen et al. (2000), where a residual cathepsin D activity of about 10% was reported after a treatment of 60 s at 99°C. These differences may be due to the different assay system applied by Larsen et al. (2000), where hemoglobin was used as a substrate that was incubated at 37°C for 16 h. These assay conditions (temperature, time) were probably not suitable for quantitatively analyzing cathepsin D activity.

Ultra-high-temperature treatments of ≥135°C are used to ensure a milk shelf life of 8 to 12 wk (German Milk Production Act, 2000). Under these conditions (140°C for 10 s), no residual cathepsin D activity was determined.

In accordance with the existing literature, cathepsin D was found to resist temperature treatments that are applied during standard milk processing, such as thermization (57 to 68°C for up to 30 s). The residual activity of cathepsin D as an endopeptidase may cause quality defects within resulting cheese products due to the enzymatic hydrolysis of milk proteins. An effect of cathepsin D on casein-derived peptide patterns in dairy products was shown for quark (Hurley et al., 2000) and feta cheese (Larsen et al., 2000). However, it remained unclear whether cathepsin D–mediated hydrolysis of milk proteins affected the taste of the product.

Inactivation constants were calculated for the interpretation of the determined inactivation of cathepsin D and for applicability in milk heating processes (Baur et al. 2015). The inactivation rate constant $K_d$ describes the direct transition of active enzyme to inactivated enzyme in a first-order reaction without any intermediate states (Sadana, 1988). The higher the $K_d$, the faster the cathepsin D was inactivated (Table 3). As expected, $K_d$ increased with higher temperatures, from $6.9 \times 10^3$ s$^{-1}$ at 60°C to $2.36 \times 10^5$ s$^{-1}$ at 80°C. No cathepsin D activity ($E_t$) was determined at 90 to 140°C and, thus, no inactivation rate was calculated. Based on the inactivation rate constant $K_d$, the half-life ($T_{1/2}$) and the decimal reduction time ($D_0$ value) of cathepsin D were calculated. The decimal reduction time is defined as the time necessary for a 90% reduction of the initial activity at a certain temperature $\vartheta$ (Deeth, 2021). Based on the $D_0$ value, appropriate heating processes can be chosen. The $T_{1/2}$ and $D_0$ of cathepsin D decreased with increasing temperature. At 60°C, $D_0$ was 5.6 min, whereas at 80°C, $D_0$ decreased to 10 s, showing that cathepsin D seemed to be highly susceptible to temperature treatments >60°C. Nevertheless, thermization of raw milk is not sufficient if complete cathepsin D inactivation is desired in a dairy product of interest.

Inactivation rate constants were used to calculate the time needed to reduce cathepsin D activity by 99% at every temperature investigated. Accordingly, a line of equal effect was created (Figure 3) to facilitate decision-making for cathepsin D inactivation. It is obvious from Figure 3 that HTST treatments inactivate cathepsin D insufficiently, whereas other common heating strategies achieve cathepsin D inactivation easily. Compared with other potentially relevant peptidases from Pseudomo-

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_d$ (s$^{-1}$)</th>
<th>$T_{1/2}$ (s)</th>
<th>$D_0$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>$6.9 \times 10^3$</td>
<td>$1.0 \times 10^2$</td>
<td>$3.3 \times 10^2$</td>
</tr>
<tr>
<td>65</td>
<td>$4.1 \times 10^4$</td>
<td>17</td>
<td>56</td>
</tr>
<tr>
<td>72</td>
<td>$1.1 \times 10^5$</td>
<td>6.1</td>
<td>20</td>
</tr>
<tr>
<td>75</td>
<td>$3.3 \times 10^5$</td>
<td>5.4</td>
<td>18</td>
</tr>
<tr>
<td>80</td>
<td>$2.4 \times 10^5$</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>90</td>
<td>ND$^1$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>95</td>
<td>ND$^1$</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>140</td>
<td>ND$^1$</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^1$Not determinable.
nas spp. or plasmin, cathepsin D is less resistant to elevated temperatures and will be inactivated.

**Effect of Cathepsin D Activity on the Formation of Bitterness Within Model Fresh Cheese**

Cathepsin D preferentially cleaves between hydrophobic amino acids, such as phenylalanine or leucine (Handley et al., 2001), resulting in partly hydrophobic peptides. Although a hydrophobic peptide does not necessarily have to be bitter, hydrophobicity as well as certain amino acids in a terminal peptide position might favor the development of a bitter taste (Zhao et al., 2016). A possible effect of cathepsin D activity on the bitter taste impression has not yet been shown and, therefore, was investigated in this study. A cathepsin D concentration of 0.4 µg mL<sup>-1</sup> was determined by Larsen et al. (1996). This corresponded to an estimated volumetric activity of 5 pkat mL<sup>-1</sup> based on the activity and protein content of the cathepsin D preparation used. Therefore, this activity was chosen to mimic a regular cathepsin D concentration in milk. In parallel, skim milk containing an elevated SCC (14 × 10<sup>5</sup> cells mL<sup>-1</sup>), supplied by the research station Meierenhof (Universität Hohenheim, Stuttgart, Germany), was examined for cathepsin D activity (8.9 ± 0.1 pkat mL<sup>-1</sup>). During udder inflammation, immune cells are recruited and the SCC in the milk increases. Therefore, it can be used as an indicator of a cow’s health status and its milk quality. When the SCC is ≥4 × 10<sup>5</sup> cells mL<sup>-1</sup>, the milk is considered unsuitable for human consumption (EU Regulation No. 853; European Union, 2004). There is a connection between SCC and cathepsin D activity present in milk, which results in unwanted changes in dairy products (Li et al., 2014). Thus, a cathepsin D activity of 10 pkat mL<sup>-1</sup> was applied to the model fresh cheese to mimic activity in milk with an elevated SCC. Any external and internal proteolytic activity within the model fresh cheese had to be avoided; therefore, UHT milk was used for the simulated fermentation, and this milk was acidified using GDL instead of a common starter culture. Additionally, the dissolved cathepsin D preparation was sterile filtered before application to avoid contamination. The amount of cathepsin D used before sterile filtration was adjusted to the desired activity after the filtration step. A microbiologically safe product was prepared by these means for sensorial evaluation. No intrinsic cathepsin D activity could be determined within the UHT skim milk used. In comparison to fermentation with a conventional starter culture, the pH was lowered faster with GDL, as the latter dissociated directly without experiencing a lag phase (Lucey et al., 1998). However, a final pH of 4.8 to 4.9 was reached after 18 to 19 h at 23°C, which is comparable to starter culture fermentation. A model fresh cheese with a protein content of around 6% was obtained by removing the whey protein fraction. Residual cathepsin D activity of 40 to 50% was determined in the whey fractions. As reported previously by Larsen et al. (1996), cathepsin D is part of the whey protein fraction and is partly removed from the model fresh cheese during centrifugation.

The model fresh cheese was stored at 4°C for around 20 h and then used for the sensorial evaluation. A panel of 11 to 15 people were not able to distinguish the cathepsin D–spiked model fresh cheeses from the control (GDL) model fresh cheese in either of the triangle tests, which were done on different days. Significance ($P < 0.01$ or $P < 0.05$) was not reached in these sensorial evaluations of the model fresh cheeses with 5 or 10 pkat mL<sup>-1</sup> cathepsin D (see Supplemental Table S1; https://figshare.com/s/5b62ad9c7186ef87396a). Most of the testers tasted slight bitterness within the samples, regardless of the presence of cathepsin D. The taste of GDL changes from sweet to sour and bitter as its dissociation progresses (Parke et al., 1997). After 19 h, complete dissociation of GDL was assumed and, therefore, a certain contribution of GDL to the slight bitterness within the model fresh cheeses was postulated. However, an effect of cathepsin D could be excluded because the bitter taste was experienced equally in all GDL-acidified model fresh cheeses.

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**Figure 3.** Time- and temperature-dependent inactivation of cathepsin D (isolate from bovine spleen) by 99% in skim milk plasmin and *Pseudomonas* peptidases in milk (modified from Stoeckel et al., 2016). Time and temperature ranges of the most important sample treatments for dairy products are indicated. LTLT = low-temperature, long-time; St. = sterilization; HT = high temperature.
The bitter peptides were extracted from the model fresh cheeses and analyzed via HPLC-MS/MS. Sebald et al. (2018) identified 17 key bitter peptides in fresh cheeses in a sensoproteomics approach that combined meta literature research with a taste dilution analysis and MS. The peptides in the model fresh cheeses of this study were analyzed via HPLC-MS/MS and the results revealed no detectable peptide concentrations or concentrations below the limit of quantification (<LOQ) for the peptide sequences analyzed (Table 4). Most of the detectable (but still <LOQ) peptides were derived from β-CN. However, peptides occur naturally in milk due to proteolytic activity within a cow’s udder. All of those peptides initially present originated from casein fractions, although β-CN contains the most putative cleavage sites for cathepsin D (Baum et al., 2013). No increase in the bitter peptide concentration was detected between the control and cathepsin D–spiked model fresh cheeses. Thus, we assume that some of the peptides were initially present within the UHT skim milk used for the model fresh cheese production. Additionally, the 2 bitter peptides with the greatest impact on bitter taste perception according to Sebald et al. (2018), MAPKHKEMPFPKYPVEPF and ARHPHPHLSFM, were not detectable in any of the samples.

Apart from the general findings of this study, some points should be considered. First, these findings were achieved by using cathepsin D isolated from bovine spleen. However, studies in the literature generally used spleen-isolated cathepsin D to produce antibodies to detect cathepsin D in milk. Thus, cathepsin D must be identical in both preparations. Second, several proteolytic enzymes are generated and present during the fermentation of milk. We cannot exclude the possibility that synergistic effects with other peptidases before processing or during fermentation with a starter culture or the application of rennet might result in the formation of bitter peptides. Even if cathepsin D was mostly removed along with the whey protein fraction, the bitter peptides would have already been generated. Based on these findings, starter cultures strains should be carefully selected and combined.

**CONCLUSIONS**

In accordance with existing data, cathepsin D activity was found to overcome low-temperature heat treatments between 60 and 72°C. Raw milk is usually heated to 90 to 95°C for 3 min before using it to produce fresh cheese. Cathepsin D activity is already undetectable after heating for 5 s at 90°C. Nevertheless, even elevated cathepsin D activities of up to 10 pkat mL\(^{-1}\) did not generate bitter peptides in the model fresh cheeses. The slightly bitter taste perceived by the trained panel was also perceived in the control cheeses and could be caused by the GDL used for acidification. Otherwise, sensory panelists could not taste a difference between the control model fresh cheese and the cathepsin D–

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**Table 4. Bitter peptides derived from casein (Sebald et al., 2018) within cathepsin D–spiked model fresh cheeses**

<table>
<thead>
<tr>
<th>Derived from:</th>
<th>Peptide sequence</th>
<th>Control</th>
<th>5 pkat mL(^{-1})</th>
<th>10 pkat mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>αs1-CN</td>
<td>VAPFPEVPGKE</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>VFGEKVNEL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>DIQKM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>EIIVPS(PHOS)VEQK</td>
<td>ND</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>IQKEDVPS</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>β-CN</td>
<td>TQPVVVPPFLQPE</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>MAPKHKEMPFPKYPVEPF</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td></td>
<td>LHLPLP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
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<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
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<tr>
<td></td>
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<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
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<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
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<td>FFSDKIAK</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>YQQPVAL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ARHPPHPHLSFM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>AIPPKKNQDKTEIPT-INTIASGEPT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>INTIASGEPT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

1<LOQ = below the limit of quantification; ND = not determinable.

**Bitter Peptide Profile of Cathepsin D–Spiked Model Fresh Cheese**

The bitter peptides were extracted from the model fresh cheeses and analyzed via HPLC-MS/MS. Sebald et al. (2018) identified 17 key bitter peptides in fresh cheeses in a sensoproteomics approach that combined meta literature research with a taste dilution analysis and MS. The peptides in the model fresh cheeses of this study were analyzed via HPLC-MS/MS and the results revealed no detectable peptide concentrations or concentrations below the limit of quantification (<LOQ) for the peptide sequences analyzed (Table 4). Most of the detectable (but still <LOQ) peptides were derived from β-CN. However, peptides occur naturally in milk due to proteolytic activity within a cow’s udder. All of those peptides initially present originated from casein fractions, although β-CN contains the most putative cleavage sites for cathepsin D (Baum et al., 2013). No increase in the bitter peptide concentration was detected between the control and cathepsin D–spiked model fresh cheeses. Thus, we assume that some of the peptides were initially present within the UHT skim milk used for the model fresh cheese production. Additionally, the 2 bitter peptides with the greatest impact on bitter taste perception according to Sebald et al. (2018), MAPKHKEMPFPKYPVEPF and ARHPHPHLSFM, were not detectable in any of the samples.

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spiked model fresh cheeses. The MS analysis supported the absence of bitterness within the model fresh cheeses.

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