MODIFICATION OF BOVINE MILK PROTEIN SYSTEM BY TRANSGLUTAMINASE

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Abstract

In this study the effect of transglutaminase (tgase) on individual bovine milk proteins in skim milk samples was investigated with a kinetic approach in skim milk samples. Raw and preheated (80 °C/ 5 minutes) samples were incubated with tgase with 2 different enzymes with the protein ratios of 1:10 and 1:20 for up to 4 hours at 3 different incubation temperatures (4, 30 and 42 °C). It was found that tgase had no effect on the native whey proteins. The highest reaction constant value (k) was found for κ-casein, and it was followed by β-casein and α-casein, respectively in heated and unheated skim milk samples. The crosslinking reaction was almost completed within the first 5 minutes at 42 °C for κ-casein. The Ea value (activation energy) of β-casein (29 kj/mol) was found to be lower than that of α-casein (45 kj/mol). On the other hand, when the kinetic parameters were evaluated, it is postulated that tgase can be used for cheese manufacturing (30 °C, at 1:10 E:P ratio) and yoghurt production (42 °C, at 1:20 E:P ratio). This approach will be investigated in further studies.

Keywords: milk, proteins, transglutaminase, cross-linking

TRANSGLUTAMİNAZ İLE İNEK SÜTÜ PROTEİN SİSTEMİNİN MODİFİKASYONU

Özet


Anahtar kelimeler: süt, proteinler, transglutaminaz, çapraz bağlama

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INTRODUCTION

Transglutaminase (tgase, E.C. 2.3.2.13, protein-glutamine γ-glutamyl-transferase) catalyses the acyl transfer reaction between γ-carboxyamide groups of peptid-bound glutamine residues (acyl donor) and the primary amino groups in a variety of amine compounds (acyl acceptor), including peptide-bound ε-amino groups of lysine residues. As a result of crosslinking of glutamine and lysine residues, high molecular weight protein polymers are formed (1).

There are a lot of researches on the application of tgase to modify the functional properties of milk proteins. Caseins are the major proteins in milk. It is well known that tgase have high reactivity on caseins. It is possibly caused by the open physical structure of the caseins. (2-7).

It is reported that the globular whey proteins are unsuitable substrate for tgase-induced crosslinking in their native state. Some modifications, especially denaturation is required in their structure (2, 3, 8, 9). On the other hand it is reported that the milk whey powder proteins (α-lactalbumin and β-lactoglobulin) show reactivity tgase without the need for any prior treatment (10).

It is observed that tgase have higher reactivity on β- and κ-casein than that of α-casein (11, 12). β-casein undergoes a temperature-dependent self-association into micelles. O’Connell and de Kruif (13) suggested that the intramolecular crosslinking of the β-casein prevents shifting of monomers from micelles, and intermolecular crosslinking in the micelles prevents dissociation with changing of the temperature.

Possible use of tgase in bovine milk processing has been studied for a few years. However, there is a lack of knowledge on the kinetics of the reactions between tgase and milk proteins. The aim of this study was to determine the susceptibility of individual bovine milk proteins towards tgase-induced crosslinking with a kinetic approach. The influence of enzyme concentration and incubation temperature on crosslinking of milk proteins with tgase was also investigated.

MATERIALS AND METHODS

Samples, Standards and Reagents

Raw bovine milk samples were obtained from a local dairy (A.O.Ç. Dairy Plant, Ankara, Turkey). All milk samples were skimmed at 5000 rpm, 7 °C for 10 minutes. Sodium azide solution was added to the skim milk samples at a ratio of 1:10000 (w/v) to prevent microbial growth. The samples were stored at 7 °C.

Microbial transglutaminase (Tgase) was supplied from Ajinomoto Foods Deutschland (ACTIVA-MP, Germany).

For identification of milk proteins and preparation of the model systems, several standards containing purified bovine milk proteins [κ-CN (#C-0406), α-CN (#C-6780), β-CN (#C-6905), α-la (#L-5385), β-lg (#L-3908)] which were purchased from Sigma (Germany) were prepared.

Acetonitrile (JT Baker, #9012) and trifluoroacetic acid (TFA) (JT Baker, #9470) and ultra-pure water were HPLC grade. All other chemicals were analytical grade.

Sample Preparation and Treatment with Transglutaminase

Preparation of reaction model systems

The standard proteins model system consisted of heated (at 80 °C for 5 minutes) and unheated β-lg, α-la and β-lg-α-la-κ-CN-αs-CN-β-CN mixture. Standard proteins were dissolved in a phosphate buffer (pH 6.8) to achieve a molar ratio of proteins that is same as the molar ratio of original bovine milk proteins. The samples were incubated with transglutaminase (E/P= 1/10 and E/P= 1/20) for up to 4 hours at 30 °C and 42 °C.

After incubation, tgase were inactivated with NH₄Cl (14) in all samples and then stored at -20 °C.

Preparation of skim milk samples

Raw skim milk and preheated skim milk (at 80 °C for 5 minutes) were incubated with tgase (E/P= 1/10 and E/P= 1/20) for up to 4 hours at 4 °C, 30 °C and 42 °C.

All samples were incubated with transglutaminase for 0, 5, 15, 30, 60, 90, 120 and 240 minutes at 4 °C, 30 °C and 42 °C, individually.

After incubation, tgase were inactivated with NH₄Cl (14) in all samples and then stored at -20 °C.

RP-HPLC

The standard proteins and the skim milk samples were diluted in solvent A and solvent B mixture (70:30) and then they were filtered through 0.45 µm cellulose acetate filter (EG0492-1) before injection.
Modification of Bovine Milk Protein System...

The Agilent 1100 series HPLC system; consisted of a quaternary pump (Agilent, G1311A), a manual injection block (Agilent, G1328B), a variable wavelength UV-detector (Agilent, G1314A), a column thermostat (Agilent, G1316A) and degasser (Agilent, G1379A) was used. The equipment was controlled by a software (Agilent ChemStation) that controlled the solvent gradient, data acquisition and data processing. A silica-based C-18 RP-HPLC column (250 mm length x 4.6 mm i.d., Agilent Zorbax 300SB-C18, particle size 5µm, pore size 30 nm) was used for protein seperation. All solutions were filtered through a nylon filter (47 mm, 0.45 µm, EG0492-1).

Chromatographic conditions were as follows; Solvents. A: Acetonitrile, water and trifluoroacetic acid in a ratio 100:900:1 (v/v/v). B: Acetonitrile, water and trifluoroacetic acid in a ratio 900:100:1 (v/v/v). Total run time; 30 minutes. Column temperature; 25 °C. Flow rate; 1.0 mL/minute. Detection wavelength; 220 nm. Injection volume of final sample solution; 20 µL.

A solvent gradient programme started at 20% of solvent B, and it was generated immediately after sample injection by increasing gradually to the proportion of solvent B to 46% at the end of the run. Then it was returned to the initial conditions in 2.4 minutes.

RESULTS

Model Systems

When heated β-lg solution incubated with tgase at 42 °C and 1:10 enzyme protein ratio, it was observed that denatured β-lg cross-linked with tgase whereas native β-lg did not react with tgase (Figure 1a). Figure 1a shows that all of the denatured β-lg (denatured complex) cross-linked at the end of the 120th minute of the reaction while the peak areas of native form of β-lg were stable.

Peak areas of α-la did not change and denatured α-la did not form during heat treatment at 80 °C for 5 minutes heat treatment. α-la found to be stable to the heat treatment and unsuitable substrate for cross-linking reactions. The peak area of α-la did not change during the tgase incubation (Figure 1b). The process temperatures for cheese and yogurt productions are 30 and 42 °C, respectively. The study was repeated at these temperatures to determine optimum enzyme concentration and incubation time for tgase reaction.

Standard protein mixture (β-lg-α-la-κ-CN-αs-CN-β-CN) was incubated with tgase at 30 and 42 °C. Evaluatuation of RP-HPLC chromatograms showed that, the protein profiles were almost the same for the combination of enzyme concentrations and reaction temperatures of [1:20]/42 °C and [1:10]/30 °C for 90 minutes (P<0.01) (Figure 2).

The difference between peak areas of the proteins for 90 minutes and 120 minutes reaction times was found to be insignificant for both of the combinations of enzyme concentrations and reaction temperatures of [1:20]/42 °C and [1:10]/30°C (p>0.05) (Figure 3 and 4). According to the results, it is postulated that tgase can be used for cheese manufacturing (30 °C, 1:10 E:P ratio) and yogurt production (42 °C, 1:20 E:P ratio). It was also found that 90 minutes was sufficient as incubation time with tgase for both of the combinations.
The effect of transglutaminase (tgase) on major bovine milk proteins was also investigated in skim milk samples by a kinetic approach.

The RP-HPLC chromatograms were evaluated for calculation of the kinetic parameters. The peak areas in the chromatograms which give information about the concentration of each protein fraction were calculated. During the crosslinking between the proteins catalyzed by tgase, a decrease in the peak area was observed. It means that the concentration of native protein fraction decreased with crosslinking during the reaction with tgase.

The rate constant (k), activation energy (E_a) and initial reaction rate were used as kinetic parameters. The protein concentration versus incubation time graphics were used for calculation of k values and initial rates at three different incubation temperatures. The E_a was calculated from the slope of the plot of lnk versus 1/T.

According to the well-known enzyme kinetics, it was used the rate equations as follows:

\[-r = \frac{d[c]}{dt} = k_c^n\]  \hspace{1cm} (eq.1)

\[-\ln r = \ln k + n \ln c\]  \hspace{1cm} (eq.2)

When the estimated \( \ln r \) was evaluated versus \( \ln c \) and the reaction order was found to be almost 1.0. Thus it was assumed that the reaction followed first-order kinetics.

According to this assumption, the rate equations were as follows:

\[-\frac{dc}{dt} = kc\]  \hspace{1cm} (eq.3)

\[c = c_0 e^{kt}\]  \hspace{1cm} (eq.4)

The first situation given in Figure 5a showed that major whey proteins (β-lactoglobulin and α-lactalbumin) were not suitable substrates for tgase in their undenatured (native) state. The variation of RP-HPLC peak areas of each whey protein during the incubation could not be evaluated because they were denatured by heat treatment before incubation with tgase. It is well known that the decreasing of peak area of denatured whey proteins is caused by heat-denaturation process. For this reason the interactions between tgase and whey proteins denatured by RP-HPLC method could not observed. The same situation was also observed during the evaluation of κ-casein peaks because of the interactions between denatured whey proteins and κ-casein in heat-treated samples.
All RP-HPLC peak areas were calculated for each major protein fraction and they were converted to the protein concentration in skim milk samples. The rate constants (k) of the crosslinking reaction were estimated for 2-different E:P ratios, 3 different incubation temperatures of proteins in skim milk samples with or without preheat treatment. Calculated k values were given in Table 1.

It was observed that the rate constant of the major bovine casein fractions increased with increasing incubation temperature and E:P ratio. As shown in Table 1 the highest k value was found for κ-casein, and it was followed by β-casein and α-casein, respectively in unheated and heated skim milk samples. However, the reactivity of tgase on κ-casein and α-casein increased in heat-treated skim milk samples while it decreased for β-casein. The κ-casein fraction of all samples reacted with tgase in 5 minutes, and then its peak could not be detected in the chromatograms. This can be explained as that κ-casein reacts with tgase first and faster than that of other caseins. It is observed that the κ-casein peaks were detectable up to 5 minutes at 42°C, up to 15 minutes at 30°C and up to 240 minutes at 4°C at 1:10 E:P ratio.

It is assumed that the most reactive substrate for tgase was κ-casein. The initial rate and E_a parameters were not calculated for κ-casein as it reacted within first 5 minutes.

As expected the initial cross-linking rate of the proteins increased with the increasing ratio of the E:P (Table 2).

Table 1. Rate constant values (k, 1/min) of skim milk samples (Cn; -casein, Cn; -casein, Cn; -casein, -1/20 and -1/10; E:P ratio, H---; preheated at 80°C/5 minutes)

<table>
<thead>
<tr>
<th>T (K)</th>
<th>Casein fractions</th>
<th>277.15</th>
<th>303.15</th>
<th>315.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>κCn-1/20</td>
<td>0.0065</td>
<td>0.0716</td>
<td>0.1452</td>
<td></td>
</tr>
<tr>
<td>κCn-1/10</td>
<td>0.0096</td>
<td>0.0771</td>
<td>0.165</td>
<td></td>
</tr>
<tr>
<td>αCn-1/20</td>
<td>0.0005</td>
<td>0.0026</td>
<td>0.0044</td>
<td></td>
</tr>
<tr>
<td>αCn-1/10</td>
<td>0.0009</td>
<td>0.0038</td>
<td>0.0049</td>
<td></td>
</tr>
<tr>
<td>βCn-1/20</td>
<td>0.0017</td>
<td>0.0054</td>
<td>0.0066</td>
<td></td>
</tr>
<tr>
<td>βCn-1/10</td>
<td>0.0027</td>
<td>0.0067</td>
<td>0.0099</td>
<td></td>
</tr>
<tr>
<td>HκCn-1/20</td>
<td>0.0094</td>
<td>0.1387</td>
<td>0.268</td>
<td></td>
</tr>
<tr>
<td>HκCn-1/10</td>
<td>0.1985</td>
<td>0.2393</td>
<td>0.3328</td>
<td></td>
</tr>
<tr>
<td>HαCn-1/20</td>
<td>0.0004</td>
<td>0.0018</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>HαCn-1/10</td>
<td>0.001</td>
<td>0.0031</td>
<td>0.0039</td>
<td></td>
</tr>
<tr>
<td>HβCn-1/20</td>
<td>0.0013</td>
<td>0.0045</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>HβCn-1/10</td>
<td>0.0021</td>
<td>0.0057</td>
<td>0.0063</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Initial rates (µg/min) of the skim milk samples (42-, 30-, 4-; incubation temperatures as °C, -1/20 and -1/10; E:P ratio, H42, H30, H4-; the samples were preheated at 80°C).

<table>
<thead>
<tr>
<th>Samples</th>
<th>α-Cn</th>
<th>β-Cn</th>
</tr>
</thead>
<tbody>
<tr>
<td>42-1/20</td>
<td>0.025±0.001</td>
<td>0.025±0.002</td>
</tr>
<tr>
<td>42-1/10</td>
<td>0.026±0.002</td>
<td>0.025±0.002</td>
</tr>
<tr>
<td>H42-1/20</td>
<td>0.017±0.001</td>
<td>0.017±0.001</td>
</tr>
<tr>
<td>H42-1/10</td>
<td>0.026±0.002</td>
<td>0.025±0.002</td>
</tr>
<tr>
<td>30-1/20</td>
<td>0.016±0.001</td>
<td>0.019±0.001</td>
</tr>
<tr>
<td>30-1/10</td>
<td>0.023±0.002</td>
<td>0.026±0.001</td>
</tr>
<tr>
<td>H30-1/20</td>
<td>0.012±0.001</td>
<td>0.015±0.001</td>
</tr>
<tr>
<td>H30-1/10</td>
<td>0.017±0.001</td>
<td>0.018±0.001</td>
</tr>
<tr>
<td>4-1/20</td>
<td>0.003±n.a.</td>
<td>0.005±n.a.</td>
</tr>
<tr>
<td>4-1/10</td>
<td>0.006±n.a.</td>
<td>0.009±n.a.</td>
</tr>
<tr>
<td>H4-1/20</td>
<td>0.006±n.a.</td>
<td>0.005±n.a.</td>
</tr>
<tr>
<td>H4-1/10</td>
<td>0.006±n.a.</td>
<td>0.007±n.a.</td>
</tr>
</tbody>
</table>
As the E:P ratio increased from 1:20 to 1:10, the effect of incubation (reaction) temperature on initial reaction rate of α-casein and β-casein decreased at 42 °C and 30 °C. It can be explained as lower concentration of tgase was required for higher reaction temperatures. The initial rates at 4 °C was found to be lowest, as expected. The lower enzyme content requires to crosslinking of milk proteins with tgase relatively, the higher incubation temperature was applied to the skim milk samples. It means that the E:P ratio can be used as 1:20 for incubation at 42 °C (i.e. yoghurt process) while as 1:10 for incubation at 30 °C (i.e. cheese manufacture). It can be explained as the reaction rates are almost same for the combination of enzyme concentrations and reaction temperatures of [1:20]/42 °C and [1:10]/30 °C.

On the other hand the difference of initial reaction rate caused by incubation temperatures at 42°C and 30°C was found to be more negligible for β-casein than α-casein. The $E_a$ values of bovine caseins (β-casein and α-casein) found to be between 27-45 kJ/mol (Table 3).

Table 3. Activation energy ($E_a$) (kJ/mol) of the unheated skim milk samples (αCn; α-casein and βCn; β-casein; -1/20 and -1/10; E:P ratio).

<table>
<thead>
<tr>
<th>Casein fractions</th>
<th>$E_a$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Cn-1/20</td>
<td>45.31±2.21</td>
</tr>
<tr>
<td>α-Cn-1/10</td>
<td>36.31±1.98</td>
</tr>
<tr>
<td>β-Cn-1/20</td>
<td>29.08±1.11</td>
</tr>
<tr>
<td>β-Cn-1/10</td>
<td>26.59±1.42</td>
</tr>
</tbody>
</table>

The $E_a$ value of β-casein was found to be lower than that of α-casein. This possibly means that β-casein was more suitable substrate than α-casein. Furthermore the effect of E:P ratio on $E_a$ for β-casein was negligible while it was found to be significant for α-casein ($P<0.05$). It is suggested that less energy was needed for crosslinking reaction for β-casein.

It was found that κ-casein was the most susceptible casein fraction to tgase-induced crosslinking. Additionally, β-casein was more attractive to enzymatic crosslinking than α-casein.

**CONCLUSION**

It is suggested that tgase can be used for cheese-making practice at relatively low temperatures and at higher E:P ratio. On the other hand, it also can be used in yoghurt production contrarily at higher temperature with relatively low E:P ratio. This kinetic approach will contribute to the future researches on tgase utilization in fermented milk processing.

**Acknowledgement**

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**REFERENCES**