NEW METHODS FOR THE DETERMINATION OF RENNIN ACTIVITY WITH $\kappa$-CASEIN

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Most milk casein is in the form of a stable colloid called micelle. It clots, however, under certain conditions. For example, when it is attacked by rennin (chymosin; EC 3.4.4.3), rennin cleaves in the first step $\kappa$-casein (1) which plays a key role in stabilizing casein micelle and milk starts precipitating as $\kappa$-casein is decomposed. The cleavage occurs at only one peptide bond between phenylalanine and methionine (2). Products of the enzymatic hydrolysis are glycopeptide from the C-terminal side and para-$\kappa$-casein from the N-terminal side (3). Several methods have been presented to determine the rennin activity on either whole casein or $\kappa$-casein. The most widely accepted method measures the time required to obtain visible particles of casein in the milk flowing at a constant rate (4). Similar methods are based on the measurement of turbidity, viscosity, or precipitation in agar-casein gel (5,6). These methods are, however, more or less indirect because they measure the secondary reaction brought about by rennin. Other methods have been devised to measure directly the reaction products or $\kappa$-casein. Glycopeptide can be obtained as a trichloroacetic acid soluble protein (7). As it does not contain any aromatic amino acid (8), the amount is estimated from the total nitrogen content or the absorption at 217 nm after time consuming dialysis (9). Precipitation with calcium or pH adjustment was tried to measure para-$\kappa$-casein formed (10). One of the problems with this method comes from the fact that the substrate molecules normally associate together in S–S bond linkage. Therefore, there is always a possibility of coprecipitating unreacted $\kappa$-casein. It is also unreliable to measure $\kappa$-casein by determining the remaining capacity of stabilizing $\alpha_s$-casein without dissociating $\kappa$-casein complex. There are other unique methods which determine the number of peptide bonds hydrolyzed with 2,4,6-trinitrobenzene sulfonic acid (11) and with pH stat (12). Recently, a synthetic peptide was used for estimating rennin activity (13).
ducts as far as rennin is regarded as a milk clotting enzyme.

Our methods, based on the determination of para-κ-casein, overcame all of the disadvantages found in the previous methods and made it possible for the first time to calculate accurate rennin units. Urea and mercaptoethanol were used to completely dissociate κ-casein complex after rennin reaction was over. Urea was recrystallized from alcohol and the solution was passed through an ion exchange resin column before it was mixed with buffer.

Rennin solution (0.06%) and κ-casein solution (1.5%) were prepared in 0.025N Tris-HCl buffer, pH 7.0. Reaction was started in a centrifuge tube by mixing 0.1 ml of κ-casein solution with 0.01 ml of rennin solution at 35°C and was stopped at various time intervals by adding 0.4 ml of 0.01 M Tris-citrate buffer, pH 7.7, containing 4.5 M urea and 0.3 % mercaptoethanol at 5°C. One tube was prepared containing the same buffers without κ-casein as a blank for UV absorption measurement. Duplicate tubes were prepared for 0 min reaction. Half milliliter of 0.01 M Tris-citrate buffer, pH 7.7, containing 4.5 M urea and 2 N NaCl was added to one of them, followed by the addition of 1 ml of the same buffer containing 4.5 M urea, 0.3 % mercaptoethanol and 20 mg CM-cellulose. The other one was treated in the same way as the rest of the tubes to which 0.5 ml of 0.01 M Tris-citrate buffer, pH 7.7, containing 4.5 M urea and 1 ml of the same buffer containing 4.5 M urea, 0.3 % mercaptoethanol and 20 mg CM-cellulose were added. They had been allowed to stand for 30 min at room temperature with intermittent shaking before they were centrifuged at 2,000 rpm for 10 min. Absorption at 280 nm was measured against the above blank. Decrease in the absorption was due to para-κ-casein bound by CM-cellulose and the amount of para-κ-casein was calculated with our predetermined absorption coefficient, 1.257, of 0.1 % solution in the same buffer. In the DEAE-cellulose method, DEAE-cellulose replaced CM-cellulose in the above method and imidazole-HCl buffer, pH 7.0, was used in stead of Tris-HCl buffer. Increase in absorption due to unbound para-κ-casein was measured to calculate rennin activity. After UV absorption measurement, the supernatant solution was put back into the centrifuge tube. Then, 0.2 ml of 5 N NaCl solution was added to recover the bound proteins and the whole solution was centrifuged again. An aliquot of the supernatant solution was analyzed by the following gel electrophoresis. A lower column (0.5 × 5 cm) of 7.5 % polyacrylamide gel containing 6 M urea (pH 8.7) was prepared according to the method of Reisfeld et al. (14). Twenty microliters of the above supernatant solution were placed on the column with the equal volume of 4 % agarose solution in 1 M Tris-HCl buffer, pH 8.7, containing 4.5 M urea and small amount of Bromphenol blue. After they were well mixed, gel formation was completed at 5°C. Then, 0.5 ml of 2 % agarose solution in the same buffer was placed on the sample gel and gelled at 5°C. After electrophoresis, the gel was stained with Coomassie blue for 30 min. After destaining, 5 mm upper gel covering para-κ-casein band was sliced and put into a test tube to be melted in 2 ml water at 90°C. The tube
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was placed in a water bath kept at 34°C and 1 ml of methanol was added to disperse the staining dye completely. Absorption at 580 nm was measured against water. Standard para-\( \kappa \)-casein was prepared in our laboratory and run in the same way to obtain a standard curve for determining the amount of para-\( \kappa \)-casein formed in the rennin reaction. A straight curve was obtained in the range of 0–23 ug.

Table 1. Rennin action on \( \kappa \)-casein. Two rennin assays were performed under similar conditions. One assay mixture was analyzed by both the CM-cellulose and the electrophoresis methods. The other one was analyzed by the DEAE-cellulose method.

The value of 12,000 was taken as the molecular weight of para-\( \kappa \)-casein.

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>( A_{280} )</th>
<th>Para-( \kappa )-casein formed (( \mu )mole)</th>
<th>( A_{280} )</th>
<th>Para-( \kappa )-casein formed (( \mu )mole)</th>
<th>( A_{280} )</th>
<th>Para-( \kappa )-casein formed (( \mu )mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.888</td>
<td>0.000</td>
<td>0.957</td>
<td>0.000</td>
<td>0.055</td>
<td>0.000</td>
</tr>
<tr>
<td>0</td>
<td>0.778</td>
<td>0.015</td>
<td>0.201</td>
<td>0.015</td>
<td>0.069</td>
<td>0.000</td>
</tr>
<tr>
<td>10</td>
<td>0.665</td>
<td>0.025</td>
<td>0.316</td>
<td>0.029</td>
<td>0.101</td>
<td>0.013</td>
</tr>
<tr>
<td>20</td>
<td>0.558</td>
<td>0.033</td>
<td>0.418</td>
<td>0.035</td>
<td>0.132</td>
<td>0.025</td>
</tr>
<tr>
<td>30</td>
<td>0.535</td>
<td>0.039</td>
<td>0.476</td>
<td>0.040</td>
<td>0.170</td>
<td>0.040</td>
</tr>
<tr>
<td>40</td>
<td>0.485</td>
<td>0.043</td>
<td>0.506</td>
<td>0.044</td>
<td>0.175</td>
<td>0.042</td>
</tr>
<tr>
<td>50</td>
<td>0.515</td>
<td>0.045</td>
<td>0.535</td>
<td>0.044</td>
<td>0.153</td>
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<tr>
<td>60</td>
<td>0.432</td>
<td>0.046</td>
<td>0.535</td>
<td>0.044</td>
<td>0.183</td>
<td>0.045</td>
</tr>
</tbody>
</table>

Table 1 shows that the results obtained by the present three methods are very similar. The presence of small difference between the control and 0 min reaction indicates that \( \kappa \)-casein used in this assay was contaminated with small amount of para-\( \kappa \)-casein. The pH condition in the mixing experiment with ion exchange cellulose may be slightly changed depending upon the substrate used. Precipitation was observed around 30 min when about 30% of the substrate was decomposed. Thereafter, the reaction began to slow down. The precipitate apparently consisted of not only para-\( \kappa \)-casein but also \( \kappa \)-casein. It was concluded that these methods are simple and accurate to determine the rennin activity on \( \kappa \)-casein which can be expressed by the unit of catalyzing the formation of one micromole para-\( \kappa \)-casein per min. In addition, the good reproducibility leaves the expectation that the rennin unit obtained by the present methods is better for expressing the milk clotting activity than that obtained by the previous milk clotting method. It is also possible to investigate effects of various reaction conditions by the present methods such as pH, temperature, substrate concentration, and presence of other milk proteins.

REFERENCES