Caseinolytic Activity of Fruit Extract from Opuntia ficus-indica on Bovine, Caprine, and Ovine Sodium Caseinates

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The rates and extents of hydrolysis of $\alpha_S$- and $\beta$-caseins from bovine, caprine, and ovine sodium caseinates produced by an enzymatic extract of the fruit of Opuntia ficus-indica, (L.) Miller were evaluated and compared with those produced by a commercial animal rennet. A mechanistic model based on a pseudo-first-order enzymatic reaction, in the presence of first-order deactivation of the enzyme, was postulated and successfully fitted to the experimental data. The animal rennet exhibited higher enzymatic efficiency than the fruit extract, irrespective of the source (i.e., bovine, caprine, or ovine) and the type (i.e., $\alpha_S$- or $\beta$-casein) of substrate. The enzymatic efficiency $(k_{cat}/K_m)$ for $\alpha_S$-casein ranged from 72 to 220 and from 43 to 65 L g$^{-1}$ h$^{-1}$, and for $\beta$-casein from 242 to 742 and from 55 to 164 L g$^{-1}$ h$^{-1}$, for the animal rennet and the enzymatic extract of O. ficus-indica, respectively. Finally, it was observed that $\beta$-casein from caprine and ovine caseinates was degraded by O. ficus-indica faster than its $\alpha_S$ counterpart, but the reverse was observed for bovine caseinate.

Introduction

Ripening of cheese is a slow and thus expensive process; therefore, there is an economic impetus toward its acceleration. Because glycolysis occurs rather quickly and lipolysis is not of crucial importance in several cheese varieties, most attempts to accelerate ripening have focused on proteolysis since coagulation is based on enzymatic breakdown of $\kappa$-casein at Phe$_{105}$–Met$_{106}$, hence leading to generation of a soluble macropeptide (that is lost in the whey during syneresis) and eventual disruption of the micelles that release $\alpha_s$- and $\beta$-caseins (otherwise confined). Hydrolysis of these latter two proteins is the main proteolytic event during cheese ripening and is often denoted as primary proteolysis; it is caused chiefly by residual rennet and produces large- and medium-sized peptides (1). Calf chymosin is still the prevailing milk coagulant used in cheesemaking worldwide. However, owing to a shortage of calf stomachs available commercially, animal rennet has been mixed with higher and higher proportions of pepsin produced by older animals. Plant rennets used traditionally in some countries exhibit milk-coagulating properties; examples include thistle (2), pineapple (3), sodom apple (4), and crude papaya (5). Nevertheless, little is known to date, on scientifically sound grounds, about the performance of such rennets when compared to that of animal rennet (1).

The genus Opuntia belongs to the family Cactaceae; it consists of 300 species of which the Opuntia ficus-indica, (L.) Miller (commonly known as Indian-fig prickly pear) has the greatest economic importance. This plant, probably native to Mexico, has adapted perfectly to the weather conditions prevailing in coastal zones and grows wild in Portugal (6). It is known for its rapid growth, good adaptation to poor soils and low requirement for water; in addition, it propagates naturally by simply dropping its pads on the ground (7). These characteristics make such plant an interesting subject of research aiming at bioindustrial applications (6, 8).

Most traditional Portuguese cheeses (e.g., Serra da Estrela, Serpa, and Azeitão) have for centuries been successfully manufactured using a plant rennet (Cynara cardunculus, L.), and the fruit of O. ficus-indica is able to coagulate bovine, caprine, and ovine milks (6). It is therefore of interest to test such fruit either as a coagulant substitute or as an accelerator of cheese ripening. The aim of this work was, thus, to quantitatively assess the caseinolytic activity of partially purified extracts of O. ficus-indica fruit upon bovine, caprine, and ovine milk caseinates, using as reference a commercial animal rennet.

Materials and Methods

Preparation of Caseinate Feedstocks. Caseinates from whole bovine (Cachena breed), caprine (Serrana breed), and ovine (Bordaleira breed) were prepared via isoelectric precipitation of the corresponding milks according to the method described by Sousa and Malcata (9). The lyophilized caseinates were kept at $-30^\circ$C until experimental proteolytic hydrolysis was in order. Substrates for these assays were prepared by dissolving 10 g of each type of caseinate separately in 1 L of 100 mM phosphate buffer (pH 6.5) at 30 $^\circ$C. To inhibit microbial...
Preparation of Fruit Extract. Crude enzymatic extract of O. ficus-indica was obtained from the unripe wild fruit (collected in Charneca da Cotovia, Sesimbra, Portugal) according to the method reported by Teixeira et al. (6) and further purified by fractionation following Teixeira et al. (10). This fruit extract was then stored at −40 °C until the assays were performed.

Determination of Protein Concentration. The protein concentrations of the fruit extract and of the commercial rennet solution containing 25% chymosin and 75% pepsin (Naturen-Stab 230, from Chr. Hansen, Denmark) were determined by the Bradford method (11).

Enzymatic Hydrolysis of Substrates. Buffered solutions (pH 7.0) of fruit extract (0.37 g protein L\(^{-1}\)) and animal rennet (0.33 g protein L\(^{-1}\)) were added at a ratio of 0.526 mL of enzyme solution to 10 mL of caseinase buffered solution (pH 6.5), and the experimental mixture was kept, under gentle stirring, in a water bath thermostated at 30 °C. Aliquots were taken at 0, 3, 8, 24, 48, and 72 h and immediately mixed (1:1, v/v) with sample buffer containing mercaptoethanol (0.2 M) and urea (8.2 M) to inactivate the enzymes and hence stop the hydrolysis reaction. All analyses were run in duplicate. These samples were kept at −30 °C until electrophoretic analysis.

Electrophoretic Analysis. Urea-polyacrylamide gel electrophoresis was performed according to the method of Andrews (12) with the modifications set forth by Shalabi and Fox (13). The gels were stained with Coomassie Blue G-250 using the procedure of Blakesley and Boezi (14), followed by staining with deionized water. Quantification of intact \( \alpha \)- and \( \beta \)-caseins was done by densitometry using a GS-700 imaging densitometer and the Molecular Analyst software (BioRad, Hercules CA).

Mathematical Modeling. The experimental data pertaining to hydrolysis of both caseins were first simulated under the assumption of Michaelis–Menten kinetics for the enzyme-mediated reaction in a batch, well-stirred apparatus, coupled with the assumption that both substrates (i.e., \( \alpha \)- and \( \beta \)-caseins) compete for the same active site of the enzyme(s), viz.

\[
\frac{-dC_i}{dt} = \frac{v_{\text{max},i} C_i}{K_{m,i} + C_i}; \quad i = \alpha, \beta; \quad j = b, c, o
\]  

(1)

where \( C_i \) is the concentration (in g L\(^{-1}\)) of substrate \( i \) (where subscript \( i \) denotes \( \alpha \)- or \( \beta \)-casein), and subscript \( j \) denotes bovine (b), caprine (c), or ovine (o) sodium caseinate; \( t \) is hydrolysis time (in h); \( v_{\text{max},i} \) is reaction rate (in g L\(^{-1}\) h\(^{-1}\)) under full saturation of enzyme by substrate \( i \); and \( K_{m,i} \) is the Michaelis–Menten constant for substrate \( i \) (in g L\(^{-1}\)). However, statistical analysis of the preliminary fits to the experimental data showed that saturation was never attained within the experimental range of interest, so eq 1 was accordingly simplified to

\[
\frac{-dC_i}{dt} = \frac{v_{\text{max},i} C_i}{K_{m,i} C_i}; \quad i = \alpha, \beta; \quad j = b, c, o
\]  

(2)

Eventual occurrence of an asymptotic plateau for \( C_i \) at values below unity suggested that inactivation (thermal or, more likely, protease-driven) of the enzyme(s) might occur simultaneously with the enzyme-mediated reaction (because the equilibrium conversion of such hydrolysis reaction approaches 100%). Therefore, a mass balance to active enzyme was considered, based on first-order kinetics, according to

\[
\frac{-dC_E}{dt} = k_{\text{cat},E} C_E; \quad i = a, f
\]  

(3)

where \( C_E \) is concentration (in g L\(^{-1}\)) of enzyme; \( k_{\text{cat},E} \) is inactivation rate constant (in h\(^{-1}\)); and subscript \( i \) denotes animal rennet (a) or fruit extract (f).

Combination of eqs 2 and 3 yields

\[
\begin{align*}
\frac{C_{a,0} - C_a}{C_{a,0}} &= 1 - \exp\left\{\frac{k_{\text{cat},a} C_{E,0} (k_{\text{cat},a} C_{E,0})}{K_{m,a} K_{E,a}} \left(\exp(-k_{E,a} t) - 1\right)\right\}; \\
\frac{C_{f,0} - C_f}{C_{f,0}} &= 1 - \exp\left\{\frac{k_{\text{cat},f} C_{E,0} (k_{\text{cat},f} C_{E,0})}{K_{m,f} K_{E,f}} \left(\exp(-k_{E,f} t) - 1\right)\right\};
\end{align*}
\]

(4)

which will hereafter be termed Model I, and where \( C_{i,0} \) denotes initial concentration (in g L\(^{-1}\)) of substrate \( i \); \( k_{\text{cat},i} \) denotes kinetic constant (in h\(^{-1}\)) for consumption of substrate \( i \); and \( C_{E,0} \) denotes initial concentration of enzyme. Only two values of \( K_{E} \) were obviously considered in Model I, one for fruit extract and another for animal rennet.

A second model (termed Model II) was also considered in this study, which assumes that the rate of inactivation of enzyme depends on the type of substrate (\( \alpha \)- or \( \beta \)-casein; \( i \)) and its source (bovine, caprine, or ovine; \( j \)). In this case, twelve distinct values of \( K_{E} \) were considered in Model II, corresponding to all combinations of source type of substrate, and nature of enzyme.

Results and Discussion

The experimental data obtained for the extent of hydrolysis of \( \alpha \)- and \( \beta \)-caseins by the fruit extract and the commercial rennet are plotted in Figure 1; the curve provided by the fit of Model I to the data is overlaid in this plot. Statistical analysis of the two models (note that Model I is nested in Model II) indicated that Model I is statistically better, at the 0.1% level of significance (see Table 1 for data pertaining to O. ficus-indica), so it will be used for discussion hereafter. This statistical analysis is also consistent with the fact that, if inactivation of the enzyme were first order (and hence unimolecular), it should be independent of both the type (i.e., \( \alpha \)- or \( \beta \)-casein) and the source (i.e., bovine, caprine, or ovine milk) of the substrate.

The best estimates for the kinetic parameters are provided in Table 2. The enzyme activity of the animal rennet decreased faster than that of the fruit extract, as implied by higher values of \( k_{f} \) for the former enzyme; the corresponding half-lives for the fruit and animal enzymes were 11.72 and 2.78 h, respectively. Table 2 shows also that the animal rennet exhibited higher enzymatic efficiency toward hydrolysis of the substrates than the fruit extract, because of the higher values of the ratio \( k_{\text{cat},f}/K_{m,f} \) for animal rennet. This result explains the differences in shape between the theoretical curves pertaining the two enzymes. For all substrates, the extent of hydrolysis brought about by animal rennet increased faster during the initial stages of reaction (owing to higher values of \( k_{\text{cat},f}/K_{m,f} \) but leveled off earlier (owing to a higher value of \( k_{f} \)) than for hydrolysis brought about by the enzymatic extract of O. ficus-indica. Some researchers have also reported that the proteolytic activity
sodium caseinate from bovine (\textbf{-}) and caprine (\textbf{bold -}), and ovine (\textbf{very bold -}) milk by (i) fruit extract (Opuntia ficus-indica) or (ii) commercial animal rennet. The theoretical fit for Model I is also represented for bovine (-), caprine (bold -) and ovine (very bold -) caseinates. Figure 1. Experimental data (duplicated experiments) for extent of hydrolysis \((C_{\text{f}0} - C)/C_{\text{f}0}\) of (a) \(\alpha_s\)-casein and (b) \(\beta\)-casein in sodium caseinate from bovine (\textbf{C}), caprine (\textbf{bold C}), and ovine (\textbf{very bold C}) milk by (i) fruit extract (Opuntia ficus-indica) or (ii) commercial animal rennet. The theoretical fit for Model I is also represented for bovine (-), caprine (bold -) and ovine (very bold -) caseinates.

Table 1. Residual Sum of Squares Analysis, Encompassing Models I and II, for the Caseinolytic Activity of Fruit Extract (Opuntia ficus-indica, (L.) Miller)

<table>
<thead>
<tr>
<th>source of variability</th>
<th>sum of squares</th>
<th>degrees of freedom</th>
<th>mean square</th>
<th>F-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>extra parameters</td>
<td>0.0765</td>
<td>5</td>
<td>0.0153</td>
<td>3.643</td>
<td>0.006</td>
</tr>
<tr>
<td>Model I</td>
<td>0.2296</td>
<td>55</td>
<td>0.0042</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model II</td>
<td>0.3061</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Best Estimates of the Kinetic Parameters for Model I Describing the Hydrolysis of \(\alpha_s\)- and \(\beta\)-Caseins in Bovine, Caprine, and Ovine Sodium Caseinates (Values Indicated Were Fitted to Duplicated Data)

<table>
<thead>
<tr>
<th>source of caseinate</th>
<th>substrate</th>
<th>(k_{\text{cat}}/K_m) (L g(^{-1}) h(^{-1}))</th>
<th>(k_E) (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine</td>
<td>Opuntia ficus-indica Fruit Extract</td>
<td>(\alpha_s)-casein 63.03</td>
<td>(\beta)-casein 54.70</td>
</tr>
<tr>
<td>caprine</td>
<td>(\alpha_s)-casein 42.70</td>
<td>(\beta)-casein 163.76</td>
<td>0.03</td>
</tr>
<tr>
<td>ovine</td>
<td>(\alpha_s)-casein 65.62</td>
<td>(\beta)-casein 98.14</td>
<td></td>
</tr>
</tbody>
</table>

Conclusions

The enzymatic extract from O. ficus-indica fruit exhibited significant caseinolytic activity on \(\alpha_s\)- and \(\beta\)-caseins in sodium caseinate obtained from bovine, caprine and ovine milk. The final degrees of hydrolysis were similar between the plant and the animal enzymes, but the hydrolysis rate profiles were distinct. Therefore, the fruit extract is apparently a good substitute for animal rennet, as it exhibits both clotting and caseinolytic activities.
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(10) Teixeira, G.; Santana, A.; Batista, I.; Pais, M. S.; Clemente, A. Extracts multienzimáticas obtidos a partir de material vegetal do género Opuntia e sua utilização industrial; proteases cistênicas com carácter ácido-moderado obtidas a partir de extractos de frutos de Opuntia ficus-indica (L.) Miller, e sua utilização industrial. Provisional Portuguese Patent No. 102330, 2000, June 15.


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References and Notes


