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 α_S- and β-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., α_S- or β-casein) of substrate. The enzymatic efficiency (k_{cat}/K_m) for α_S-casein ranged from 72 to 220 and from 43 to 65 L g^{-1} h^{-1}, and for β-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 β-casein from caprine and ovine caseinates was degraded by *O. ficus-indica* faster than its α_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 κ-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 miceles that release α_S- and β-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-clotting properties; examples include thistle (*2*), pineapple (*3*), soddom 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 °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 °C. To inhibit microbial

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growth, sodium azide was added to a final concentration of 0.1%(w/v).

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 caseinate 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 destaining with deionized water. Quantification of intact α_S- and β-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 Michaëlis−Menten kinetics for the enzyme-mediated reaction in a batch, well-stirred apparatus, coupled with the assumption that both substrates (i.e., α_S- and β-caseins) compete for the same active site of the enzyme(s), viz.

$$\left(-\frac{dC_i}{dt} = \frac{\nu_{max,i}\frac{C_i}{K_{m,i}}}{1+\frac{C_\alpha}{K_{m,\alpha}}+\frac{C_\beta}{K_{m,\beta}}}\right)_j;\ i=\alpha,\beta;\ j=\text{b, c, o} \qquad (1)$$

where C_i is the concentration (in g L^{-1}) of substrate i (where subscript i denotes α_S- or β-casein, and subscript j denotes bovine (b), caprine (c), or ovine (o) sodium caseinate); t is hydrolysis time (in h); $\nu_{max,i}$ is reaction rate (in g L^{-1} h^{-1}), under full saturation of enzyme by substrate i; and $K_{m,i}$ is the Michaëlis−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

$$\left(-\frac{dC_i}{dt} = \frac{\nu_{max,i}}{K_{m,i}}C_i\right)_j;\ i=\alpha,\beta;\ j=\text{b, c, o} \qquad (2)$$

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

$$\left(-\frac{dC_E}{dt} = k_{E,l}C_E\right);\ l=\text{a, f} \qquad (3)$$

where C_E is concentration (in g L^{-1}) of enzyme; k_E is inactivation rate constant (in h^{-1}); and subscript l denotes animal rennet (a) or fruit extract (f).

Combination of eqs 2 and 3 yields

$$\left(\begin{array}{l}\frac{C_{\alpha,0}-C_\alpha}{C_{\alpha,0}} = 1-\text{EXP}\left\{\left(\frac{k_{cat,\alpha}C_{E,0}}{K_{m,\alpha}k_{E,l}}\right)(\exp(-k_{E,l}t)-1)\right\}\\ \frac{C_{\beta,0}-C_\alpha}{C_{\beta,0}} = 1-\text{EXP}\left\{\left(\frac{k_{cat,\beta}C_{E,0}}{K_{m,\beta}k_{E,l}}\right)(\exp(-k_{E,l}t)-1)\right\}\end{array}\right)_j;$$

$$l=\text{a, f};\ j=\text{b, c, o} \qquad (4)$$

which will hereafter be termed Model I, and where $C_{i,0}$ denotes initial concentration (in g L^{-1}) of substrate i; $k_{cat,i}$ 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 (α_S- or β-casein; i) and its source (bovine, caprine, or ovine; j). In this case, twelve distinct values of $k_{E,l}$ 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 α_S- and β-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., α_S- or β-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_E 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_{cat}/K_m 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_{cat}/K_m) but leveled off earlier (owing to a higher value of k_E) than for hydrolysis brought about by the enzymatic extract of *O. ficus-indica*. Some researchers have also reported that the proteolytic activity

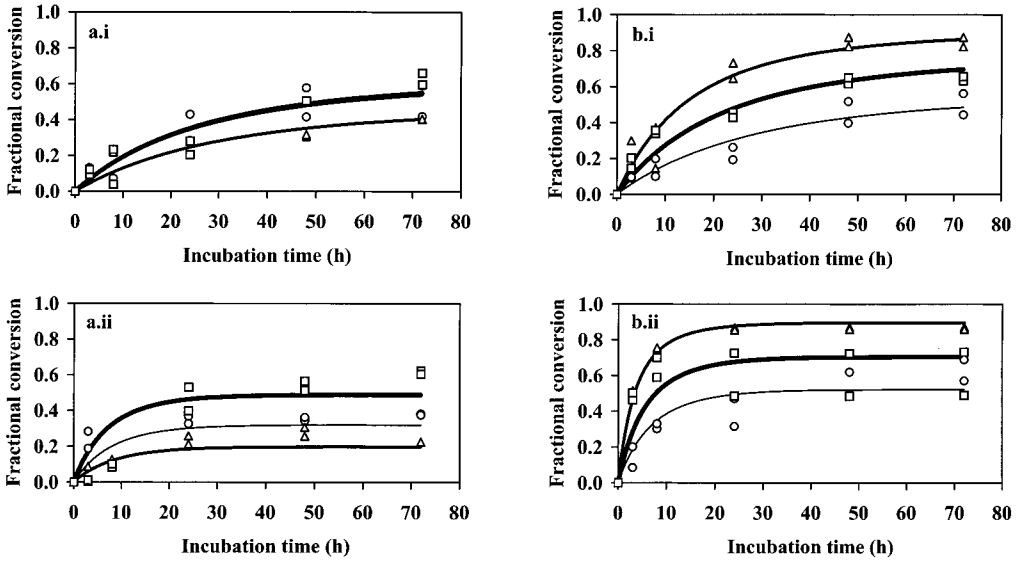

Figure 1. Experimental data (duplicated experiments) for extent of hydrolysis $(C_{i,0} - C_i)/C_{i,0}$, of (a) α_S-casein and (b) β-casein in sodium caseinate from bovine (○), caprine (△), and ovine (□) milk by (i) fruit extract (*Opuntia ficus-indica*) or (ii) commercial animal rennet. The theoretical 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)

source of variability	sum of squares	degrees of freedom	mean square	*F*-ratio	*P*
extra parameters	0.0765	5	0.0153	3.643	0.006
Model II	0.2296	55	0.0042		
Model I	0.3061	60			

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

		kinetic parameters	
source of caseinate	substrate	k_{cat}/K_m (L g^{-1} h^{-1})	k_E (h^{-1})
	Opuntia ficus-indica Fruit Extract		
bovine	α_S-casein	63.03	
	β-casein	54.70	
caprine	α_S-casein	42.70	0.03
	β-casein	163.76	
ovine	α_S-casein	65.62	
	β-casein	98.14	
	Commercial Animal Rennet		
bovine	α_S-casein	126.94	
	β-casein	241.55	
caprine	α_S-casein	72.61	0.11
	β-casein	742.03	
ovine	α_S-casein	220.45	
	β-casein	399.42	

of animal rennet was higher than that of *C. cardunculus* (*2*) or of *Centaurea calcitrapa* (*15*).

The hydrolysis rate profiles effected by the fruit extract on α_S-casein obtained from bovine and ovine caseinates indicate that this enzyme acts on both substrates at similar rates because of the identical progress reaction curves and the similar values for the ratio k_{cat}/K_m. In the case of caprine caseinate, a lower value for the pseudo-first-order rate constant (k_{cat}/K_m) was noticed (see Figure 1.a.i and Table 2). Consequently, the extents of hydrolysis by 72 h of reaction were virtually equivalent for bovine and ovine caseinates but higher than that obtained for their caprine counterpart. The proteolytic behavior of the fruit extract on the three caseinates was also different from that exhibited by the animal rennet. Animal rennet acted preferentially on ovine caseinate, followed by bovine and finally caprine (see Figure 1.a.ii). The final extents of hydrolysis of α_S-casein from bovine and caprine sources brought about by the fruit extract were higher than by the animal rennet; for ovine caseinate they were identical for α_S-casein.

The hydrolysis rates profiles by the fruit extract on β-casein were identical for the three sources of caseinates and also identical to that of animal rennet (see Figures 1.b.i-ii); both enzymes exhibited the highest enzymatic efficiency on caprine caseinate (i.e., the highest value for k_{cat}/K_m), followed by ovine and then bovine caseinate (see Table 2). In fact, there are differences between the primary structures of β-caseins in the three milk types (e.g., β-caseins from caprine and ovine sources are similar but slightly different from that from bovine source), and there are also differences in the degree of phosphorylation, all of which might explain differences in the hydrolysis rate profiles of β-casein (*16*, *17*).

Furthermore, the fruit extract exhibited higher enzymatic efficiency toward β- than α_S-casein, from both caprine and ovine sources (the ratios of k_{cat}/K_m for β-casein to those of α_S-casein were 3.83 and 1.50 for caprine and ovine caseinate, respectively); this is in agreement with reports pertaining to the caseinolytic activity of enzymes from *Cynara* spp. upon caprine and ovine caseinates (*2, 18*). These researchers also claimed that the rate of hydrolysis by those plant enzymes is dependent on the substrate, with a preference for peptide bonds linking residues with bulky hydrophobic side chains.

Conclusions

The enzymatic extract from *O. ficus-indica* fruit exhibited significant caseinolytic activity on α_S- and β-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.

Notation

B	substrate blank test
C	substrate concentration (g L^{-1})
v	reaction rate (g L^{-1} h^{-1})
k_{cat}	reaction rate constant (h^{-1})
k_E	enzyme inactivation rate constant (h^{-1})
K_m	Michaëlis−Menten kinetic constant (g L^{-1})
t	time (h)

Subscripts

a	animal rennet
b	bovine sodium caseinate
c	caprine sodium caseinate
E	enzyme
f	fruit extract
i	type of substrate
j	type of milk
l	type of enzyme
max	maximum
o	ovine sodium caseinate
α	α_S-casein
β	β-casein
0	initial value

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