The effect of the crosslinking enzyme tyrosinase on gel formation and texture of acid-induced milk gels

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ABSTRACT

Caseins were crosslinked by *Trichoderma reesei* tyrosinase in both raw and heat-treated skim milk. Tyrosinase-induced crosslinking was beneficial in the preparation of acidified milk gels from raw milk, but it did not have an effect on acid gels made from heat-treated milk

INTRODUCTION

Enzymes that form covalent bonds between or within proteins are potential tools in improving the texture of dairy products such as yogurt. The beneficial effect of transglutaminase (EC 2.3.2.13) in acidified milk gels is well known. Transglutaminase (TG) catalyses the formation of covalent crosslinks between glutamine and lysine residues in protein molecules.[1]

Tyrosinase (EC 1.14.18.1) is an interesting alternative to TG, as its mode of action is very different from the action of TG. In the presence of oxygen tyrosinase catalyses the hydroxylation of a monophenol to an *o*-diphenol, which is further oxidised to the corresponding *o*-quinone. Quinones are highly reactive compounds which can further react non-enzymatically with each other or with thiol and/or primary amino groups. In addition to its natural substrates tyrosine and dihydroxyphenylalanine (DOPA), tyrosinase is capable of oxidizing tyrosine residues (Fig. 1) in protein molecules, resulting in the formation of covalent tyrosine-tyrosine, tyrosine-cysteine or tyrosine-lysine cross-links.[2-4] The substrate specifity of tyrosinase is highly dependent on its origin. Tyrosinase extracted from mushroom (*Agaricus bisporus*) is known to crosslink whey proteins in model systems.[5] The tyrosinase from the fungus *Pycnoporus sanguineus* has proven to be an efficient crosslinker of casein.[6] In this work the application of a novel tyrosinase[7] from the fungus *Trichoderma reesei* in acidified milk gels is reported. The action of *T. reesei* tyrosinase is compared with the action of the some commercially available crosslinking enzymes, i.e. mushroom tyrosinase and TG.

MATERIALS AND METHODS

Enzymes

Two different tyrosinases were used in the work: 1) a commercial mushroom (*Agaricus bisporus*) tyrosinase (AgaTYR) (Fluka, Switzerland) and 2) a tyrosinase from the filamentous fungus *Trichoderma reesei* (TrTYR).[7] Tyrosinase activity was assayed using 15 mM L-DOPA (Sigma, USA) as substrate at pH 7 and room temperature according to the method of Robb.[8] Activa® MP transglutaminase (TG) preparation was obtained from Ajinomoto Co., Inc. (Japan) and further purified at VTT.

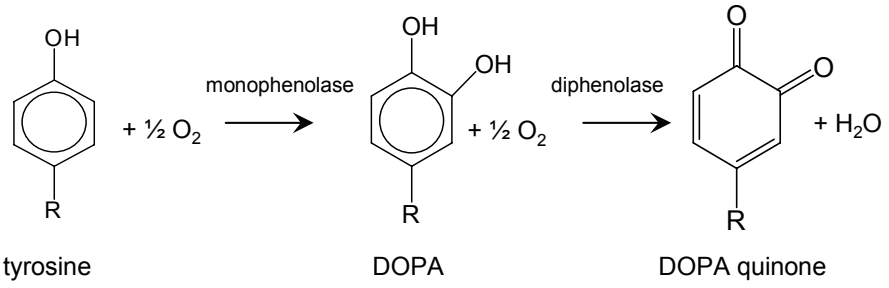

Figure 1. Oxidation of a monophenol (tyrosine) to an o-diphenol (DOPA) and an o-quinone (DOPA quinone) by tyrosinase.

The activity of the transglutaminase preparation was assayed according to Folk[9] using 0.03 M N-carbobenzoxy-L-glutaminylglysine as substrate. Enzyme activities are expressed as nanokatals (nkat). One nanokatal is defined as the amount of enzyme activity that converts one nmol of substrate per second in the assay conditions.

Enzyme treatments

Raw or heated (5 min at 90 °C) skim milk was incubated with TrTYR, AgaTYR or TG for 1 h at 40 °C. The dosage of all enzymes was 500 nkat/g protein. After the enzyme treatment the milk samples were analysed with SDS-PAGE according to Laemmli.[10] A Bio-Rad electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) and ready-made 12% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA) were used. Protein bands were visualized by staining with Serva Blue R (Serva Electrophoresis, Heidelberg, Germany).

Preparation of acid milk protein gels

Acid milk gels were prepared from raw skim milk or skim milk heated at 90°C for 5 min. Milk was treated separately with 100 nkat/g AgaTYR, 100 nkat/g TrTYR or 25 nkat/g TG at 40°C for 1 h prior to acidification. After the enzyme treatment, all samples were cooled down to 30 °C and acidified by the addition of 1.2% D-(+)-gluconic acid δ-lactone (GDL, Sigma, USA). Acidified milk samples were incubated at 30 °C for 20 h. The final pH of the samples was about 4.6.

Rheological measurements

Small deformation

The structure formation of milk gels was followed with a stress-controlled rheometer (StressTech, Reologica Instruments AB, Lund, Sweden) in dynamic oscillation mode. Samples were placed into the concentric cylinder measuring system after 20 min of GDL addition. Silicon oil was applied on the sample surface to avoid drying. All measurements were carried out at 30 °C for 20 h at a frequency of 0.1 Hz. Strain was controlled at 0.01. Results are average of two measurements for all samples.

Large deformation

The firmness of the milk gels was measured with a TA-HDi Texture Analyser (Stable Microsystems Ltd., Godalming, UK) equipped with a 5 kg load cell. Gels were tested directly after incubation at 30 °C for 20 h. Gels were deformed by penetrating a hemispherical plastic probe (∅ 1.27 cm) at a constant speed of 0.5 mm/s to a distance of 70% of the gel height. The area under the force-deformation curve (up to a depth of 8 mm) was determined and was taken as a measure of gel firmness. For each gel type four replicate samples were measured.

RESULTS AND DISCUSSION

Crosslinking of milk proteins

TrTYR crosslinked milk proteins both in raw and heated milk, which was observed in SDS-PAGE (Fig. 2, lanes 4 and 8) as a weakening of the casein bands and formation of high molecular weight polymers. Proteins were not crosslinked by AgaTYR either in raw or heated milk (Fig. 2, lanes 3 and 7). However, Thalmann and Lötzbeyer[5] reported cross-linking of α-lactalbumin by AgaTYR but crosslinking of β-lactoglobulin by AgaTYR was not possible unless a phenolic compound was present in the system as a mediator. Selinheimo et al.[11] have recently shown that TrTYR was capable of crosslinking α-casein as such, while AgaTYR was able to form crosslinks only in the presence of L-dopa. According to SDS-PAGE (Fig. 2), crosslinking of α-lactalbumin or β-lactoglobulin was not observed with either tyrosinase even if the milk was preheated at 90 °C. This suggests that tyrosinases crosslink only caseins and that preheating does not play an important role.

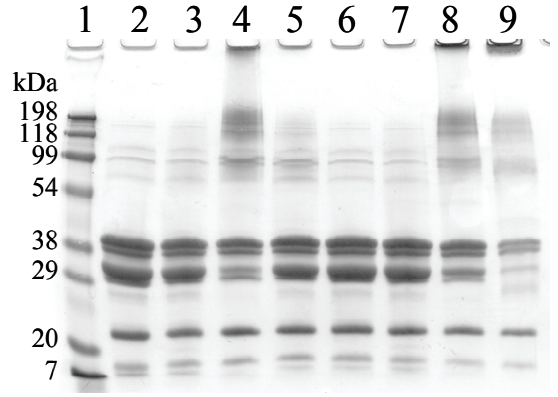

Figure 2. SDS-PAGE analysis of enzyme-treated milk: Lanes 1) molecular weight standard, 2) raw milk, 3) raw milk + AgaTYR, 4) raw milk + TrTYR, 5) raw milk + TG, 6) heated milk, 7) heated milk + AgaTYR, 8) heated milk + TrTYR; 9) heated milk + TG.

The reason for the very limited crosslinking of milk proteins in raw milk by TG (Fig. 2, lane 5) may be the presence of a low molecular weight inhibitor of TG in the milk serum.[12] When heated milk was treated with TG, extensive protein crosslinking was observed (Fig. 2, lane 9) probably due to inactivation of the inhibitor by high temperature. Mainly caseins but also β-lactoglobulin was observed to react with TG.

The two studied tyrosinases showed a large variation in their crosslinking ability. This is apparently due to differences in their substrate specificity.

Gel formation

Oscillatory rheology was used to analyse the viscoelastic behaviour of the enzyme-treated and chemically acidified raw and heated skim milk gels. Storage modulus (G′) of the samples was monitored during acidification at 30 °C for 20 h (Fig. 3). The final G′ refers to the G′ value attained after 20 h of GDL addition.

For all the samples, G′ vs time curves had similar shapes with a rapid increase in G' after gelation point. With heat-treated skim milk (Fig. 3b), acid gels showed a slightly different gel development profile than with raw milk (Fig. 3a): the onset of gel formation occured earlier and higher G' values were obtained. Final G' for enzyme-free control for heated milk was 438 Pa while for raw milk it was 17 Pa (Table 1). Heating of milk leads to many changes like denaturation of whey proteins and their subsequent interaction with casein micelles[13] as well as aggregation of micelles.[14] Heating above 70 °C causes whey protein denaturation. Some denatured whey proteins associate with micellar κ-casein via hydrophobic interactions and disulphide bonds.[15-16] Van Vliet and Keetels[17] reported that the resistance to deformation in milk gels is proportional to the number of contact points per cross-section of the network. Roefs et al.[18] reported that the storage modulus of acidified casein gels is dependent on the

number and strength of bonds between casein particles. The intermolecular cross-links formed during heat treatment of milk could be responsible for increased G' of the network.[19-22] Due to denaturation of whey proteins and their association with caseins, the gelation of heated milk starts at a higher pH (>5) than the gelation of raw milk (pH 4.8).[22] The effect of heat treatment on acid milk gel structure (rheological, textural, and microstructural) is broadly discussed in a review article by Lucey and Singh.[23]

In the case of raw milk (Fig. 3a), TrTYR treatment had a significant effect on the rate of gel formation (slope of the initial phase) for the acidified raw milk. Final G' of the gel made from TrTYR-treated raw milk was three times higher than that of the enzyme-free control (Table 1). On the other hand, AgaTYR and TG treatments in raw milk resulted in similar gel development curves and final G' values with the enzyme-free control gel. TrTYR was the only enzyme which resulted in protein cross-linking in raw milk (Fig. 2). The formation of intermolecular covalent bonds by TrTYR before and during acidification resulted in higher G'-values than in the other gels made from raw milk.

In the case of heated milk, TG treatment led to a firmer gel with significantly higher G' compared with enzyme-free control gel, while AgaTYR and TrTYR treatments did not have any positive effect on G'. The crosslinking efficiency of TG in heated milk was verified with SDS-PAGE (Fig. 2). A dosage of 25 nkat TG/g protein was enough to result in an almost two-fold increase in final G' (Table 1).

It has been reported that a small number of covalent crosslinks introduced by TG results in increased final G′,[24] firmness,[25-26] and breaking strain[27] of acid milk gels. It was expected that TrTYR would have a positive impact on final G' of the gels made from heated milk as the extent of crosslinking was the same in raw and heated milk (Fig. 2). Apparently, the tyrosinase-induced crosslinks in heated milk did not have a positive impact on gel formation. The reason for this needs to be further studied.

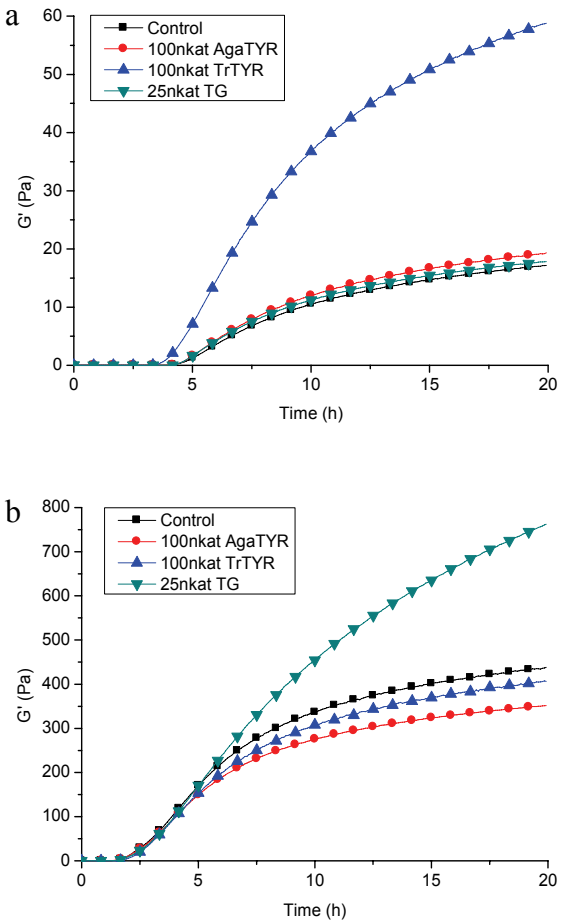

Figure 3. Storage modulus (G´) of a) raw and b) heated milk gels acidified with GDL (1.2%) at 30°C. Before acidification, raw and heated skim milk samples were enzyme treated (100 nkat TrTYR, 100 nkat AgaTYR or 25 nkat TG) for 1 h at 40°C.

Table 1. Effect of enzyme treatment of raw or heated milk on the final G' values of acidified gels prepared from the enzyme-treated milks.

	Final G' of gels (Pa)			
	Control	100 nkat AgaTYR	100 nkat TrTYR	25 nkat TG
Raw milk	17	19	59	18
Heated milk	438	352	406	762

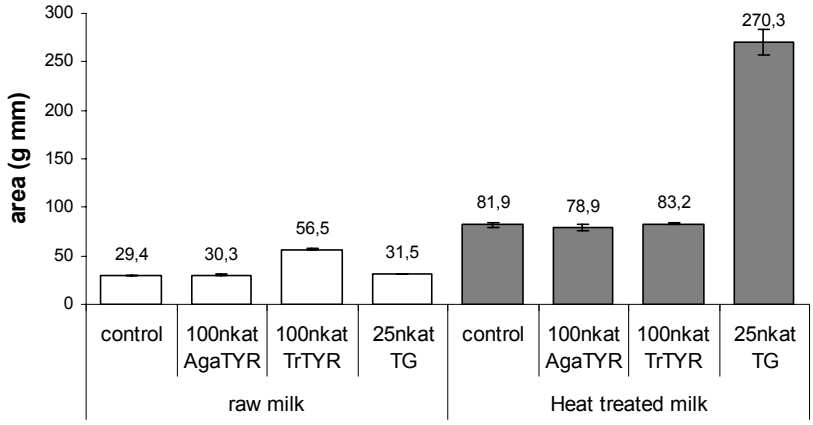

Figure 4. Firmness of chemically acidified milk gels prepared from raw and heated milk. Milk was treated with AgaTYR, TrTYR or TG for 1 h at 40°C prior to acidification.

Texture of milk gels

Texture analysis was carried out 20 h (at 30°C) after the GDL addition for all gels. The pH of the gels after 20 h acidification was about 4.6.

The area under the force-deformation curve (up to a depth of 8 mm) was taken as a measure of gel firmness (Fig. 4). When comparing the firmness values for raw and heated skim milk in general, it was observed that heat treatment did not have that great effect on large deformation measurements as it had on small deformation results. Lucey et al.[19] reported that heat treatment of milk with increasing temperature increased the brittleness of gels made from it. Van Vliet and Keetels[17] also showed that gel stiffness increased by a factor of 20-25, while fracture strain decreased to approximately half its value when milk was heated at 85 °C before gelation. Our firmness results indicated that although heated milk has a superior number of intermolecular bonds compared with raw milk (according to G' values), it still forms weak gels.

For the raw milk gels, it was observed that gel firmness was not significantly affected by AgaTYR or TG treatments. On the other hand, firmness of the raw milk gel treated with TrTYR was found to be two times higher than the firmness of the enzyme-free control (Fig. 4), which is very much in accordance with the final G' value for the same gel. The increased firmness was a result of cross-linking of caseins, as shown in the SDS-PAGE gel in Fig 2. TG treatment did not affect the firmness of raw milk gel probably due to inhibited protein cross-linking.

As shown in Fig. 4, heating the milk at 90 °C prior to enzyme treatment considerably increased the firmness of the gels prepared from TG-treated milk, whereas the firmness of the gels prepared from TrTYR-treated milk was almost unaffected by the preheating step. From heated milk much firmer gels were obtained with TG than with TrTYR.

On the other hand, TrTYR treatment did not result in big increase in gel firmness compared to the control heated milk gel even though protein cross-linking was as efficient as in the raw milk case. This phenomenon was also observed in small deformation measurements and will be further analysed.

When comparing the effect of two tyrosinases at the same activity level, mushroom tyrosinase did not increase the firmness of the gel as compared with the gel prepared from non-enzyme-treated milk, whereas there was a three fold

increase in the case of T. reesei tyrosinase in raw milk (Fig. 4).

CONCLUSIONS

The novel tyrosinase produced from *Trichoderma reesei*[7] induced crosslinking of caseins in raw milk, while mushroom tyrosinase and TG were ineffective. *T. reesei* tyrosinase treatment of raw milk prior to acidification resulted in increased gel stiffness. As the crosslinking ability of *T. reesei* tyrosinase, unlike that of TG, does not necessitate pre-heating of milk, this enzyme can be exploited in texture engineering of milk products in which excessive heating of the milk is undesirable.

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