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Chemical Composition and Rheological Properties of Set Yoghurt Prepared from Skimmed Milk Treated with Horseradish Peroxidase

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Summary

The aim of this work is to determine the impact of an enzymatic treatment on the fermentation and rheological properties of set yoghurt prepared from skimmed milk. Skimmed bovine milk was treated with horseradish peroxidase added at the level of 645 U per g of proteins in the presence (addition level of 7.8 mmol per L of milk) or absence of ferulic acid as a cross-linking agent, and used to prepare set yoghurt with commercial direct vat set starter culture. The evaluation showed that the treatment of skimmed milk with horseradish peroxidase enhanced its apparent viscosity, and storage and loss moduli. The prepared yoghurt contained protein, fat and total solids at 3.49–3.59, 0.46–0.52 and 15.23–15.43 %, respectively, had titratable acidity of 0.83–0.88 %, and no significant difference in the composition was found among the yoghurt samples ($p > 0.05$). Compared to the control yoghurt, the yoghurt prepared from the milk treated with horseradish peroxidase had a higher apparent viscosity, storage and loss moduli and flow behavior indices, especially when ferulic acid was added. Yoghurt samples from the skimmed milk treated either with horseradish peroxidase only or with the additional ferulic acid treatment had better structural reversibility, because their hysteresis loop area during rheological analysis was larger ($p < 0.05$).

Key words: skimmed milk, set yoghurt, horseradish peroxidase, ferulic acid, rheological properties

Introduction

Rheological properties of yoghurt products are mainly determined by their composition and production conditions. Any method disturbing the balance among milk components impacts directly on the rheological properties of the yoghurt (*1*). For example, elevation of protein content leads to increased thixotropy of the yoghurt (*2*), while incorporation of milk fat in the emulsified state results in a product with increased viscoelastic properties (*3,4*). With the increased consumers' demand for reduced-fat yoghurt, efforts have been made to improve its texture and rheology. Increase of the level of non-fat total solids level in the milk and/or the addition of some natural or synthetic gums as stabilizers are two conventional methods (*5,6*). However, the addition of stabilizers into the milk is restricted in some areas. Investigation of alternative methods to improve the quality of low-fat yoghurt has become an area of considerable research interest (*7*). Enzyme-induced protein interaction in milk is thus suggested as an applicable approach to improve the rheological properties of yoghurt (*8*). Transglutaminase can catalyze an acyl transfer reaction between glutamine and lysine residues in food proteins (*9,10*), which can lead to new intra- and/or inter-molecular cross-linking and structural or functional modification. Many studies have revealed the potential application of transglutaminase in some dairy products including yoghurt (*11,12*),

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but there is also a need to find another enzymatic approach to improve its rheological properties.

Cross-linking of tyrosine residues is found in native proteins and glycoproteins, for example in plant cell walls (*13*). Dityrosine cross-linking was also identified in wheat and it plays an important role in the formation of protein network in gluten (*14*). Dityrosine cross-linking can be formed by treating the proteins with hydrogen peroxide or peroxidase. Horseradish peroxidase, an important haeme-containing enzyme, can induce cross-linking of some proteins in the presence of H_2O_2 and a low molecular mass hydrogen donor (*15*), to form an oxidative phenolic coupling of adjacent groups and finally cross-link the proteins (*16*). Based on its ability to form cross-links in protein molecules, horseradish peroxidase might have potential applications in dairy processing.

In the present work, the potential application of horseradish peroxidase in yoghurt processing has been investigated. Skimmed bovine milk was treated with horseradish peroxidase in the presence or absence of ferulic acid, and fermented with a commercial direct vat set starter culture to prepare set yoghurt. The prepared yoghurt samples were evaluated for some chemical and rheological properties, in order to reveal the influence of treatment of skimmed milk with horseradish peroxidase on the rheological properties of the set yoghurt.

Materials and Methods

Chemicals and apparatus

Bovine milk was obtained from a local dairy producer in Harbin, Heilongjiang Province, PR China. Ferulic acid (FA) and horseradish peroxidase (HRP; EC 1.11. 1.7) were purchased from Shanghai Guoyuan Biotech Inc. (Shanghai, PR China). Direct vat set (DVS) starter culture used for yoghurt preparation was purchased from Rhodia (Melle, France) and stored at –18 °C before use. Other chemicals were of analytical grade. Highly purified water treated with Milli-Q PLUS water purification system (Millipore Corporation, New York, NY, USA) was used to prepare all buffers and solutions.

Treatment of skimmed milk and yoghurt preparation

Bovine milk was skimmed by centrifugation at 3000×*g* for 30 min according to the reference method (*17*) in laboratory. About 3.0 L of skimmed milk were heated in a water bath at 90–95 °C for 5 min, cooled to 25 °C and adjusted to pH=9.5 by adding 1 mol/L of NaOH solution. Cross-linking of the milk proteins was started by adding 3 % (by mass per volume) H_2O_2 to the skimmed milk to obtain 1.0 mL of H_2O_2 in 1.0 L of milk and by adding HRP solution to a level of 645 U per g of proteins, in the presence (7.8 mmol per L of milk) or absence of FA (*18,19*). Control milk was treated with the same procedure but without the addition of H_2O_2, HRP and FA. The whole mixture was mixed well and the reaction was carried out at 37 °C with gentle agitation for 4 h. After reaction, the treated skimmed milk was adjusted to pH=6.6 with 1 mol per L of HCl solution, heated at 90–95 °C for 5 min to inactivate HPR. Three treatment batches were carried out for each treatment. The control milk, HRP-treated milk and skimmed milk treat-

ed with both HRP and FA were assayed for their rheological properties, and used for yoghurt preparation as below.

The set yoghurt samples were prepared from the treated or control skimmed milk according to the procedure of Vargas *et al.* (*20*) with table sugar addition of 8 % (by mass per volume). After heat treatment at 90–95 °C for 5 min, about 3.0 L of milk were cooled to 42 °C, inoculated with the DVS starter (containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) at a level of 0.5 g per kg of milk, recommended by the supplier, and poured into glass containers under aseptic conditions. Each glass container of yoghurt samples had a capacity of 100 mL and held about 50 mL of the treated milk. Incubation of the yoghurt samples was carried out at 42 °C for 5 h. Then, the prepared yoghurt samples were placed at 4 °C for 36 h of storage. Three yoghurt samples were selected randomly from the bulk samples of each treatment batch as final analysis samples for future evaluation. The mean value of three analysis samples of each treatment batch was calculated and the results from three treatment batches were used in statistical analysis.

Rheological evaluation of skimmed milk and yoghurt samples

A Bohlin Gemini II rheometer (Malvern Instruments Ltd, Worcestershire, UK) was used to perform rheological analysis. Apparent viscosity of the milk and yoghurt samples was determined according to the Purwandari *et al.* (*21*) at 25 °C by using a parallel plate (60 mm diameter). A controlled shear rate ranging from 0.01 to 100 s^{-1} was applied during analysis. The samples were brought into lower plate by using a plastic spatula and the gap was filled up by lowering the upper plate down to the designated gap (500 μm). Excess samples around the edge of the plate were removed with a tissue. Storage modulus (G') and loss modulus (G'') of the samples were also measured by using parallel plates with diameter of 60 mm. During analysis, the frequency varied from 0.1 to 4 Hz at an applied strain of 0.5 % according to the method of Hemar *et al.* (*22*).

Thixotropy of the prepared yoghurt samples was evaluated using the method of Purwandari *et al.* (*21*). The samples were placed on an inset plate and subjected to high shearing at 500 s^{-1} for 1 min to reduce the structural differences among the samples. This was followed by 5 min of equilibration to allow a structural rebuilding of the samples. Flow curves were generated by measuring shear stress as a function of shear rates from 0.01 to 100 s^{-1} (up and down sweeps). Flow behaviour was described by Ostwald de Waele model:

$$\tau = K \cdot \gamma^n \qquad /1/$$

where τ is shear stress (Pa), γ is shear rate (s^{-1}), K and n are consistency factor (Pa·s^n) and flow behaviour index, respectively. Data were obtained directly from the rheometer using Rheo Win Pro v. 6.50.5.7 software (Malvern Instruments Ltd., Worcestershire, UK). Hysteresis loop area between the upward and downward flow curves of each analysis sample was also obtained by using this software.

Storage modulus (G') and loss modulus (G'') of the prepared yoghurt samples were measured by using parallel plate (60 mm diameter) in the rheometer, and the applied frequency varied from 0.1 to 10 Hz at 5 % strain (determined from an amplitude sweep at 1 Hz) as in the method of Purwandari *et al.* (*21*).

Chemical analysis of yoghurt samples

The prepared yoghurt samples were analyzed for their protein content, fat content and total solids using the Kjeldahl, Gerber and oven drying methods as described in AOAC official methods (*23*). A pH meter (model DELTA 320, Mettler-Toledo Instruments Ltd., Shanghai, PR China) was used to monitor the pH of the yoghurt samples during the fermentation period of 5 h or after 36 h of storage. Titratable acidity of the yoghurt samples was measured according to the AOAC method (*23*), and expressed as percentage of lactic acid in the samples. Approximately 10 g of the yoghurt sample was diluted with 10 mL of water before titration.

Statistical analysis

All experiments were carried out in triplicate, and the analysis carried out for each treatment batch was at least for three samples. All data obtained were expressed as mean values±standard deviation. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan's multiple range tests. SPSS v. 13.0 software (SPSS Inc., Chicago, IL, USA) and MS Excel 2003 software (Microsoft Corporation, Redmond, WA, USA) were used to analyze and report the data.

Results and Discussion

Rheological properties of skimmed milk treated with HRP

Apparent viscosity of the skimmed milk subjected to different shear rates is presented in Fig. 1. The apparent viscosity of the skimmed milk decreased as the shear rate increased due to shear thinning behaviour. Skimmed milk treated with HRP and FA had the highest apparent viscosity and the control milk had the lowest one, which
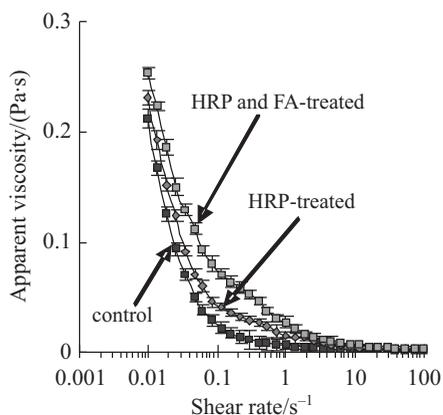
indicates that HRP treatment could enhance the apparent viscosity of the treated skimmed milk, especially when cross-linking agent FA was added. This effect was supported by the fact that the storage modulus (G') or loss modulus (G'') of skimmed milk was also influenced by treatment with HRP (Fig. 2). Skimmed milk treated with HRP and FA exhibited the highest G' or G'', while the control milk showed the lowest ones. These results indicated that modification of these rheological properties of the treated skimmed milk in the present work might be related to the modification of the main milk components, milk proteins, as Matheis and Whitaker (*24*) had confirmed that dityrosine and tertyrosine were formed during cross-linking of casein or soybean proteins by peroxidase treatment.
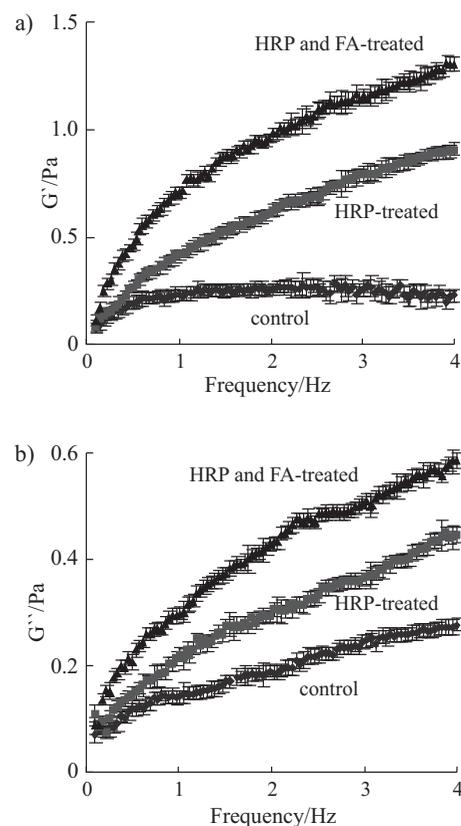

Fig. 2. a) storage modulus (G') and b) loss modulus (G'') of the skimmed milk (control) and skimmed milk treated with HRP only, or with HRP and FA

Chemical characteristics and rheological properties of yoghurt samples

Table 1 lists some chemical characteristics of the prepared yoghurt samples. The data indicate that HRP treatment in the presence or absence of FA did not exhibit any impact on protein content, fat content, total solids or final titratable acidity of the prepared yoghurt samples, as each evaluated index was at the same level without significant difference ($p>0.05$) among the yoghurt samples. Changes in the profiles of pH and titratable acidity of the yoghurt samples during fermentation period of 5 h are given in Table 2, which shows that the pH of the skimmed milk decreased, while the titratable acidity in-

Fig. 1. Apparent viscosity of the skimmed milk (control) and skimmed milk treated with HRP only, or with HRP and FA as a function of shear rate

Table 1. Main chemical composition of the prepared set yoghurt samples

Yoghurt sample	w(protein)/%	w(fat)/%	w(total solids)/%	Titratable acidity as lactic acid/%
I	$(3.59\pm0.38)^a$	$(0.52\pm0.01)^a$	$(15.43\pm0.13)^a$	$(0.85\pm0.01)^a$
II	$(3.53\pm0.64)^a$	$(0.49\pm0.01)^a$	$(15.35\pm0.19)^a$	$(0.88\pm0.01)^a$
III	$(3.49\pm0.30)^a$	$(0.46\pm0.01)^a$	$(15.23\pm0.07)^a$	$(0.83\pm0.01)^a$

I, II and III are the set yoghurt samples prepared from the skimmed milk and skimmed milk treated with HRP only, or with HRP and FA, respectively, and stored at 4 °C for 36 h. The same lowercase letters in superscript in the same column indicate that the mean values of one-way ANOVA results are not significantly different (p>0.05)

Table 2. Changes of pH and titratable acidity of the set yoghurt samples during fermentation

Fermentation time/h	Yoghurt sample	pH	Titratable acidity as lactic acid/%
1	I	6.52	0.23
	II	6.48	0.26
	III	6.30	0.24
2	I	5.84	0.32
	II	5.78	0.38
	III	5.84	0.34
3	I	5.38	0.50
	II	5.41	0.45
	III	5.39	0.48
4	I	5.00	0.56
	II	4.92	0.58
	III	4.86	0.58
5	I	4.55	0.73
	II	4.58	0.68
	III	4.54	0.73

I, II and III are the set yoghurt samples prepared from the skimmed milk and skimmed milk treated with HRP only, or with HRP and FA, respectively. The original pH and titratable acidity of the treated bovine milk were 6.58 and 0.15 %, respectively
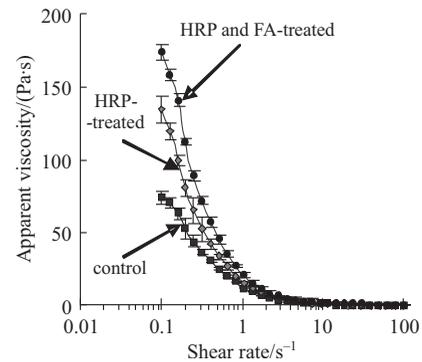

Fig. 3. Apparent viscosity of the yoghurt samples prepared from the skimmed milk (control) and skimmed milk treated with HRP only, or with HRP and FA and stored at 4 °C for 36 h as a function of shear rate
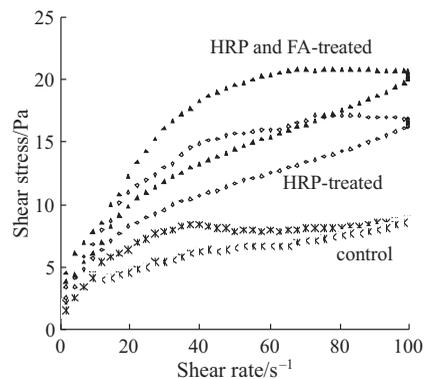

Fig. 4. Thixotropy loop of the yoghurt samples prepared from the skimmed milk (control) and skimmed milk treated with HRP only, or with HRP and FA and stored at 4 °C for 36 h

creased gradually during yoghurt fermentation. Compared to the acid production in the control yoghurt, treatment of skimmed milk with HRP or the combination of HRP and FA did not show any impact on the acid production in the yoghurt samples. These results mean that the treatment of skimmed milk had no impact on the fermentation or main chemical composition of the yoghurt samples, i.e. the different rheological properties of the prepared yoghurt samples are not the result of their different composition or acidity.

Apparent viscosity of the prepared yoghurt samples in relation to the shear rate is presented in Fig. 3. The yoghurt sample prepared from the skimmed milk treated with HRP and FA had the highest value, but that prepared from the control milk had the lowest one. Among the three prepared yoghurt samples, the obtained thixotropic hysteresis loops showed significant (p<0.05) difference (Fig. 4, Table 3). The hysteresis loop area of the yoghurt sample prepared from the skimmed milk treated with HRP and FA was the largest, while that of the yoghurt sample prepared from the control milk was the smallest (298.2 vs. 184.3 U). It had been reported that when milk proteins were cross-linked by transglutaminase (TGase; EC 2.3.2.13), hysteresis loop area was larger for the TGase-treated yoghurt in comparison with an untreated control (25), which supports the present result. Hysteresis loop was assumed to be the difference between the energy required for structural breakdown and rebuilding (26). In the present work, the yoghurt sample prepared from the skimmed milk treated with HRP only or with HRP and FA had a tendency to have better structural reversibility (viz. greater hysteresis loop area) than that prepared from the control milk. In other words, the treatment of

Table 3. Flow behaviour indices (*n*), consistency coefficient (*K*), regression coefficient (R^2) and hysteresis loop area of the prepared set yoghurt samples

Yoghurt sample	K/Pa·s^n	n	R^2	Hysteresis loop area/U
I	$(1.36\pm0.33)^a$	$(0.29\pm0.02)^a$	0.996 ± 0.19	$(184.3\pm11.1)^a$
II	$(1.80\pm0.14)^{ab}$	$(0.30\pm0.08)^a$	0.994 ± 0.28	$(250.2\pm22.0)^b$
III	$(2.07\pm0.21)^b$	$(0.30\pm0.08)^a$	0.993 ± 0.28	$(298.2\pm8.4)^c$

I, II and III are the set yoghurt samples prepared from the skimmed milk and skimmed milk treated with HRP only, and with HRP and FA, respectively, and stored at 4 °C for 36 h before analysis. Different lowercase letters in superscript in the same column indicate that one-way ANOVA results of mean values are significantly different ($p<0.05$)

skimmed milk with HRP (especially when FA was added) indeed improved the quality of the prepared set yoghurt. The data given in Table 3 also show that HRP treatment of skimmed milk had no significant influence on the resulting flow behaviour indices (*n*) of the yoghurt samples ($p>0.05$), but might have enhanced the consistency coefficient (*K*), especially when FA was added ($p<0.05$). This result was supported by the work of Truong *et al.* (*27*), in which they studied rheological changes of whey protein solution treated with TGase and found the consistency coefficient of the resulting solution to be higher.

Storage modulus (*G'*) and loss modulus (*G''*) of the prepared yoghurt samples were obviously impacted by HRP treatment of skimmed milk, as shown in Fig. 5. The yoghurt sample prepared from the skimmed milk treated with HRP and FA had the highest *G'* and *G''*, but that prepared from the control milk had the lowest ones.
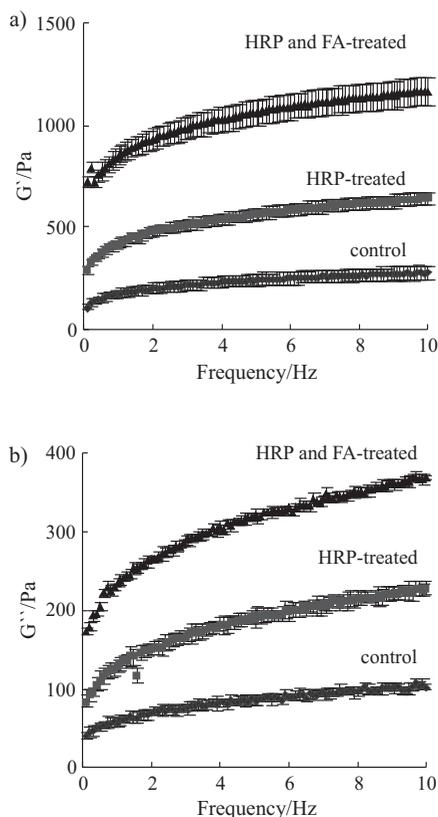
Anema *et al.* (*28*) investigated the effect of TGase-induced cross-linking of milk proteins on the viscoelastic properties of the acid gels, and found a marked increase in *G'* and *G''*. Similar to this result, the present work also showed that the treatment of skimmed milk with HRP (especially when FA was added) modified the viscoelastic properties of the prepared set yoghurt.

Different rheological properties among the prepared yoghurt samples arose from the modification of milk proteins catalyzed by HRP (especially when FA was added), which shows that this approach has potential application to improve product quality in yoghurt processing. More studies are needed to further reveal the details about the HRP-induced cross-linking of milk proteins, including the amount of peptide polymers, structural changes of the polymers and some important functional properties of the modified milk proteins.

Conclusions

Treatment of skimmed bovine milk with HRP only, or in combination with FA showed an impact on the rheological properties of the milk by enhancing its apparent viscosity, storage modulus and viscous modulus, especially when FA was added. However, the treatment did not have any influence on the main chemical composition or fermentation of the prepared yoghurt. Compared to the control yoghurt, the yoghurt prepared from the treated milk had higher apparent viscosity, storage and viscous moduli, and greater hysteresis loop area or better structural reversibility, showing that this approach might be a possible alternative to modify rheological properties of defatted set yoghurt.

Fig. 5. a) storage modulus (*G'*) and b) loss modulus (*G''*) of the yoghurt samples prepared from the skimmed milk (control) and skimmed milk treated with HRP only, or with HRP and FA and stored at 4 °C for 36 h

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