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ORIGINAL PAPER

Effect of succinylation on the functional properties of yak caseins: a comparison with cow caseins

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Abstract Yak caseins are special materials which has attracted more and more attention of researchers due to its increasing. Succinylation is a useful method of improving the functional properties of protein significantly. This study assessed the effects of succinylation on the functional properties of yak and cow caseins, including solubility, emulsifying property, water and oil absorption capacity, foaming property, and digestibility. Six succinylated levels of caseins were prepared by adding different amounts of succinic anhydride to caseins. The succinylated degree of yak and cow caseins was different when similar amounts of succinic anhydride were added. Succinylation improved solubility, emulsifying activity, water and oil absorption capacity, and reduced foam stability and digestibility of yak and cow native caseins. However, succinylation decreased foaming capacity and emulsion stability of cow caseins while increased them of yak caseins. The functional properties of caseins changed irregularly with increasing levels of succinylation. The findings of this study revealed that succinylation had different effects on the functional properties of different sources of caseins. The results will provide the basic data of functional properties of yak native caseins and succinylated yak caseins.

Keywords Succinylation · Yak caseins · Cow caseins · Functional properties

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1 Introduction

Yaks (*Bos grunniens*) from the Qinghai-Tibetan plateau are sole sources of milk of local inhabitants (Li et al. 2011). In the past years, yak milk has attracted more and more attention, due to its increasing. Yak milk is widely used to produce butter, soft and hard cheeses, yogurt, milk powder, Qula, and casein-containing products (Li et al. 2010).

Caseins, the major protein in milk, are commonly used in the food, chemical, cosmetic, and pharmaceutical industries. Yak caseins are widely used to produce high quality food ingredients and soaps, glues, leather polishing reagents, and clothing, among others. However, compared to cow caseins, yak caseins have poor solubility, which affects the other functional properties. The poor solubility of yak caseins is mostly attributed to its conformation. Yak caseins have significantly higher micellar calcium and lower inorganic phosphorus than cow caseins (Wang et al. 2013). Furthermore, the amino acid sequence of yak caseins is different than that of other caseins (Cui et al. 2012; Bai and Yin 2011; Zhang et al. 2010). Additionally, yak caseins have different composition, size, and hydration than cow caseins (He et al. 2013; Wang et al. 2013; Li et al. 2011; Li et al. 2006;). These factors contribute to the different functional properties of yak caseins.

Chemical modification is commonly used to modify the functional properties of food proteins (Matemu et al. 2011; Vidal et al. 2002; 1998). Succinylation, which is the most frequently used modification method, enhances solubility, emulsification, and foaming characteristics of proteins (Mirmoghtadaie et al. 2009; Lawal and Adebowale 2004; Achouri et al. 1998; Gruener and Ismond 1997b). Succinylation has been shown to improve the functional properties of cow caseins (Strange et al. 1993; Lakkis and Villota 1992; Schmandke et al. 1981; Schwenke et al. 1981; Hoagland 1966, 1968). However, these studies were performed with cow caseins. Could succinylation improve the functional properties of yak caseins? Could differences in composition and size of yak casein micelles affect the succinylation process?

The objective of this study was to assess the effect of succinylation on the functional properties (solubility, emulsion capacity, water and oil absorption capacity, foaming capacity and digestibility) of yak caseins. The effect of succinylation on different types of caseins (yak caseins versus cow caseins) was also discussed. The findings obtained from this study will provide the basic data of functional properties of yak native caseins and succinylated yak caseins.

2 Materials and methods

2.1 Materials

Yak and cow caseins, provided by Gansu Huaan Biotechnology, had a caseins content of 94.20 and 94.31% (*w*/*w*), respectively.

2.2 Chemical modification of caseins

Dried caseins were dissolved in distilled water at 20 mg.mL^{-1} by constant mixing at 3,000 rpm (40 °C). The pH of the solution was kept at 7.0 with 1 M NaOH. After

caseins were completely dissolved, succinic anhydride was added under constant stirring for 50 min at 42 °C. Succinic anhydride was added at different succinic anhydride/caseins ratios: 0.04:1, 0.06:1, 0.08:1, 0.1:1, 0.2:1 and 0.6:1 (g/g). The pH of the solution was initially maintained at 8.0–9.0 with 1 M NaOH; subsequently, the pH was adjusted to 7.0 with 1 M HCl. The solution was dialyzed against distilled water for 48 h at 4 °C in a dialysis bag (Oso-T8280, MD25, 8000–14000, Union Carbide Corporation, Danbury, USA) and centrifuged in a Beckman Optima XL-100 K ultracentrifuge (rotor 70Ti; Beckman Coulter, USA) at 120,000 g for 40 min at 20 °C. The resulting residue, which consisted of the modified caseins, was freeze-dried to constant weight.

2.3 Determination of the degree of modification

The degree of modification was determined by the o-phthalaldehyde (OPA) method (Dinnella et al. 2002; Malabat et al. 2001). In this experiment, 3 mL of caseins solution (0.2 $mg.mL^{-1}$) was mixed with 3 ml of OPA, which was prepared according to the method reported by Dinnella et al. (2002). After 2 min, absorbance was measured at 340 nm in a 1-cm length quartz cell (UV-2100 spectrophotometer; Beijing Beifen-Ruili Analytical Instrument Co., Ltd., Beijing, China).

The number of amino groups was calculated from an L-leucine standard curve. The percentage of amino group-modified caseins was calculated using the following formula,

$$\text{Modification degree}\,(\%) = (N_o - N_m)\Big/N_o \times 100 \tag{1}$$

where N_0 and N_m are the number of free amino groups in the unmodified and modified caseins, respectively.

2.4 Solubility

Caseins solubility was measured according to the method reported by Lawal and Adebowale (2004). Briefly, 1 g of caseins was dispersed in 100 mL of distilled water; 2.5 mL of the solution was mixed with distilled water and adjusted to pH 2–12 using either NaOH or HCl solutions (0.5–2.0 M). The final volume was 50 mL. The mixtures were mixed by a vortex shaker (QL-866, Qilinbeier instrument manufacturing Co., Ltd., Jiangsu, China) at 3,000 rpm for 1 min every 10 min for 2 h and centrifuged at 10,000×g for 35 min. Protein content in the supernatant was determined by the Kjeldahl method. Triplicate measurements were performed. Solubility was calculated using the following formula,

$$\text{Solubility}\,\% = \left(\text{Amount of protein in the supernatant}\Big/\text{Amount of protein in the sample}\right) \times 100$$

2.5 Emulsifying properties

Caseins emulsifying properties were measured by the method reported by Pearce and Kinsella (1978) with slight modifications. To prepare the emulsions, 2.0 ml of canola oil and 6.0 ml of 2 $g.L^{-1}$ caseins in water (pH 7.0) were mixed in a high-speed

homogenizer (XHF-D, Ningbo Scientz Biological Technology Co., Ltd., Zhejiang, China) at 20,000 rpm for 1 min. Subsequently, 50-μL aliquots of the emulsion were removed 0.5 cm from the bottom of the tube and dispersed in 5 mL of 0.1% (*w*/*v*) SDS. Absorbance was measured at 500 nm against a 0.1% (*w*/*v*) SDS solution blank. The emulsion was left undisturbed for 30 min. Subsequently, 50-μL aliquots were removed 0.5 cm from the bottom of the tube and dispersed in 5 mL of 0.1% (*w*/*v*) SDS. The absorbance of the solution was measured at 500 nm as described above. The emulsifying activity index (EAI, $m^2.g^{-1}$) and emulsion stability index (ESI, %) were calculated by the following formulas,

$$\mathrm{EAI}\left(\mathrm{m}^2.\mathrm{g}^{-1}\right) = 2 \times 2.303 \times A_0 \times \mathrm{dilution} \Big/ \left[C \times \varphi(1-\theta) \times 10^3\right] \tag{2}$$

$$\mathrm{ESI}\ (\%) = 100 \times A_{30} \Big/ A_0 \tag{3}$$

where C is protein concentration ($g.L^{-1}$) before emulsification, φ is optical path (0.01 m), θ is the oil volume fraction (*v*/*v*) of the emulsion (θ=0.25 here), dilution is 100, A_0 represents the absorbance at time zero and A_{30} represents the absorbance after 30 min. EAI and ESI were measured in triplicate.

2.6 Water and oil absorption capacities

Water and oil absorption capacities of caseins samples were determined according to Matemu et al. (2011). Briefly, distilled water or oil was added to caseins and mixed by a vortex shaker at 3,000 rpm for 1 min every 10 min for 30 min. The contents were allowed to stand at room temperature for 2 h and centrifuged at 3,000×*g* for 30 min. Free water or oil was removed carefully. The amount of absorbed water or oil was determined by weight difference.

2.7 Foaming properties

Foaming activity and foam stability were assessed by the method reported by Jiang and Zhao (2011) and Motoi et al. (2004) with some modifications. In this experiment, 100 mL of caseins in distilled water (1%, *w*/*v*; pH 7) was transferred to a 250-mL cylindrical glass cup and stirred at 20,000 rpm for 1 min in a high-speed homogenizer. Foam volume was measured immediately after agitation and again after 5, 10, and 30 min of setting. Foaming capacity was expressed in terms of the relative overrun; foam stability was expressed as the ratio between foam volume after 5, 10, and 30 min and the initial foam volume (0 min). Foaming capacity and foam stability were calculated using the following formulas,

$$\text{Foaming capacity} = \left(V_0 \Big/ V_a\right) \times 100 \tag{4}$$

$$\text{Foam stability} = \left(V_i \Big/ V_0\right) \times 100 \tag{5}$$

where V_0 is the foam volume at 0 min; V_i is the foam volume at 5, 10, and 30 min; and V_a is the initial liquid volume before foaming.

2.8 In vitro trypsin digestibility

In vitro trypsin digestibility of caseins was determined according to the method reported by Tang et al. (2008) with some modifications. Briefly, 50 mL of caseins dispersions (1%, *w*/*v*) in distilled water (pH 8.0) was mixed with 10 mg of trypsin powder. The mixtures were incubated at 37 °C for 0–120 min. At each time point, 4 mL of solution was transferred to a glass tube and the reaction was stopped by adding an equal volume of 20% (*w*/*v*) trichloroacetic acid (TCA). Caseins precipitates were removed by centrifugation at 10,000×*g* for 20 min. TCA-soluble nitrogen in the supernatants was determined at 280 nm.

2.9 Statistical analyses

All data were expressed as mean±SD (standard deviation) from at least three independent trials. The differences were assessed by one-way analysis of variance (ANOVA) and Duncan's multiple range tests. Statistical significance was set at $P<0.05$. PASW Statistics 18.0 software (SPSS Inc., Chicago, IL, USA) and Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) were used to analyze the data.

3 Results and discussion

3.1 Modification degree

The amount of modified lysine group in caseins was dependent on the amount of succinic anhydride added. The results revealed that the degree of modification was not linearly related to succinic anhydride/caseins ratio. The succinic anhydride/caseins ratio increased from 0.04:1 to 0.6:1 (g/g); the degree of succinylation increased from 34.62 and 38.23% to 90.00 and 87.16% in yak and cow caseins, respectively (Table 1). This was comparable to the results obtained with succinylated mung bean protein and soy protein hydrolysate. The succinylation degree of mung bean protein was approximately 80%, with a succinic anhydride/protein ratio of 0.6:1 (g/g), which was lower than that of caseins (El-Adawy 2000). Achouri and Zhang (2001) reported that the succinylation degree of soy protein hydrolysate was 88.4%, with a succinic anhydride/protein ratio of

Table 1 Succinylation extent of yak and cow caseins as a function of succinic anhydride to protein ratios. Columns with different letters are significantly different ($n=3$, $P<0.05$)

Ratio of succinic anhydride to casein (g/g)	Extent of N-succinylation (%)	
	Yak casein	Cow casein
0	0^{a}	0^{a}
0.04	34.62 ± 0.21^{b}	38.23 ± 0.22^{h}
0.06	47.21 ± 0.34^{c}	53.27 ± 0.27^{i}
0.08	57.94 ± 0.10^{d}	65.65 ± 0.35^{j}
0.1	73.33 ± 0.32^{e}	70.25 ± 0.40^{k}
0.2	85.77 ± 0.43^{f}	83.26 ± 0.29^{l}
0.6	90.00 ± 0.24^{g}	87.16 ± 0.49^{m}

50%. The succinylation degree of canola 12S globulin was 53 and 61% with a succinic anhydride/protein ratio of 0.5 and 1.0, respectively (Gruener and Ismond 1997a). Proteins have different conformations resulting in different levels of succinylation with similar succinic anhydride amount. Moreover, the difference of pH of the protein solutions can also lead to different rates of modification.

The amount of lysine amino groups in yak caseins and cow caseins measured by OPA method were 67.7 and 58.8 $mg.g^{-1}$ caseins, respectively. It suggested that the content of lysine in yak caseins is higher than that in cow's. It has been reported that lysine in yak milk is higher than that in cow milk (Li et al. 2011). Under low succinic anhydride/protein ratios, the degree of succinylation of yak caseins was lower than that of cow caseins; opposite results were obtained under high succinic anhydride/protein ratios. These results revealed that the number and reactivity of amino groups in yak and cow caseins is not similar; different steric and conformational constraints could affect the availability of free amino groups to succinylation. With increasing modification, the number of polar groups in caseins increases, which improves solubility, affects caseins conformation, and exposes previously buried amino groups. Therefore, the succinylation degree of yak caseins was higher than that of cow caseins.

3.2 Nitrogen solubility

The pH-solubility profile of caseins had a typical bell-shaped curve (Fig. 1), with a minimum solubility at pH 3. It was showed that the nitrogen solubility of native yak caseins was lower than that of native cow caseins in all cases. Yak caseins have lower solubility because of their bigger size and higher mineral content (calcium, magnesium,

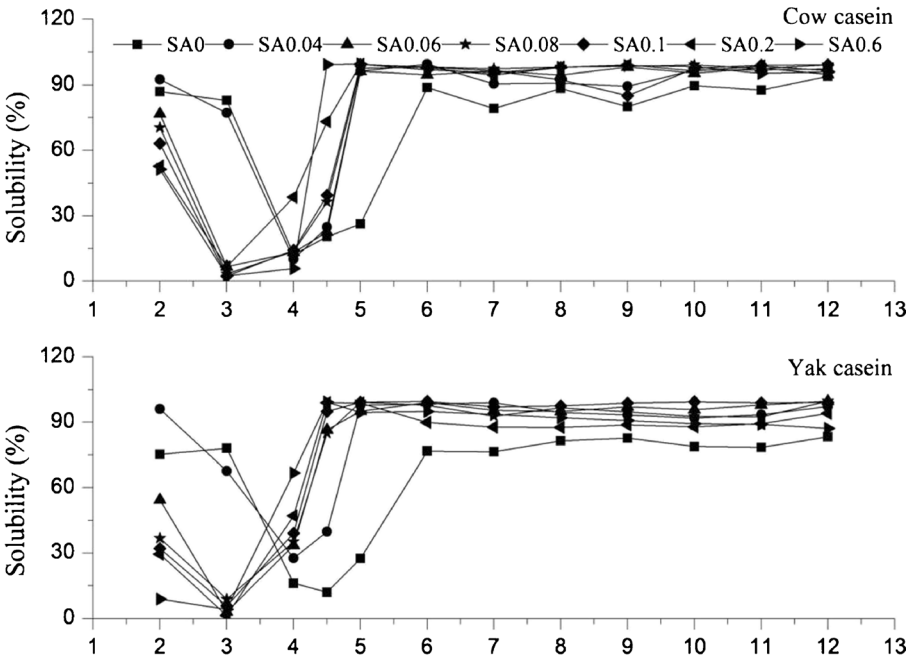

Fig. 1 Protein solubility profiles of yak casein and cow casein. *SA*: succinylated caseins (n=3)

and phosphorus), resulting in more mineral bonds and higher conformational stability than cow caseins (Wang et al. 2013; Li et al. 2011). With increasing succinylation degree, nitrogen solubility was different between succinylated yak and cow caseins, and the solubility increased at above 4 and 4.5 for cow and yak caseins respectively. However, nitrogen solubility was higher than that of the native caseins except at pH 3. After succinylation, the pH with minimal solubility changed from 4.5 to 3. The chemical modification shifts isoelectric point to lower pH value that is why minimum solubility is shifted to lower pH values (Schwenke et al. 1981). The solubility of yak caseins was similar to that of cow caseins with exhaustive succinylation in all cases. It was indicated that the solubility of yak caseins was related to pH, which agreed with the report (Liu et al. 2013).

Succinylation is a method that increases protein net charge and intra- and intermolecular electrostatic repulsion. Therefore, by promoting unfolding, reducing protein–protein aggregation, decreasing hydrogen bonding, increasing dissociation of subunits from quaternary structure, reducing isoelectric point to lower value, and increasing protein–water interactions, succinylation increases protein solubility (Achouri et al. 1998).

3.3 Emulsifying properties

The emulsifying activity of yak and cow caseins increased after succinylation (Fig. 2). With increasing degree of succinylation, the emulsifying activity of yak caseins increased and that of cow caseins increased at first and subsequently decreased except with 0.6:1 g/g succinic anhydride/caseins. The increase in cow caseins emulsifying activity was significant. However, the yak caseins emulsifying activity was not significantly different between 0.06–0.08 and 0.20–0.60 g succinic anhydride per gram of

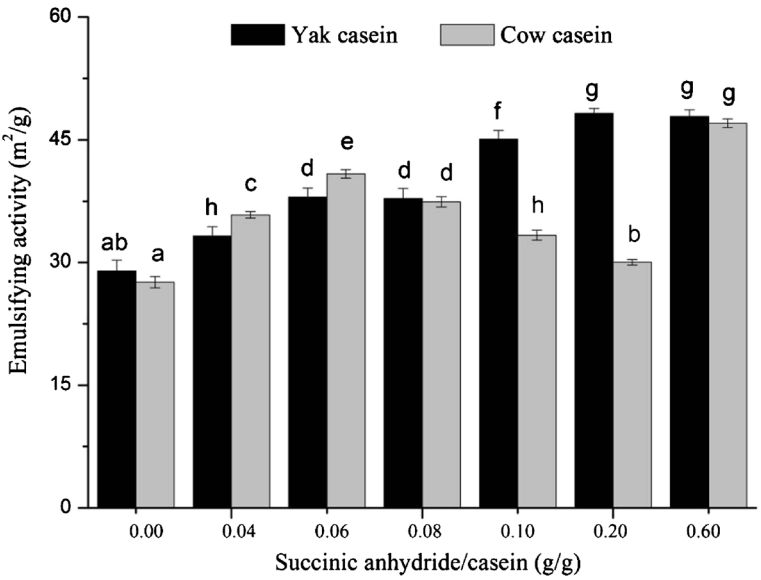

Fig. 2 Emulsifying activity of succinylated yak and cow caseins. *Columns* with *different letters* are significantly different (n=3, P<0.05)

caseins. Increased emulsifying activity after succinylation was an indication of increased solubility. As the protein became more soluble, a layer was formed around the oil droplets to facilitate their association with the aqueous phase (Mirmoghtadaie et al. 2009). Additionally, buried functional groups within the caseins micelles were exposed by succinylation, thereby increasing interactions at the protein–oil interface (Lawal and Adebowale 2004).

At <0.1 g succinic anhydride, EAI of modified and unmodified yak caseins was similar to that of cow caseins. Surface amino groups were succinylated at low succinic anhydride amounts and surface hydrophobicity decreased, contributing to an improved hydrophobic–hydrophilic balance for emulsification. When the conformation of caseins was affected by succinylation, previously buried hydrophobic regions were exposed and the balance of hydrophobic–hydrophilic was altered, resulting in a reduction in the emulsification activity of modified cow caseins. In the presence of high succinic anhydride amounts, the balance of hydrophobic–hydrophilic was restored due to increased caseins micelle dissociation and solubility, thereby increasing EAI. Yak caseins had larger size and lower solubility than cow caseins. With succinylation, the enhancement of yak caseins solubility had a more significant effect on emulsification than the balance of hydrophobic–hydrophilic.

The emulsion stability of yak caseins increased with degree of succinylation. Modified cow caseins had lower ESI values compared to unmodified cow caseins (Fig. 3). Even though an increase in solubility by succinylation should improve ESI of cow caseins, an excessive increase in the net charge might increase casein–casein repulsion, prevent casein–casein interactions so that no elastic protein film can form at the oil–aqueous phase interface, and contribute to less stable emulsions (Chan and

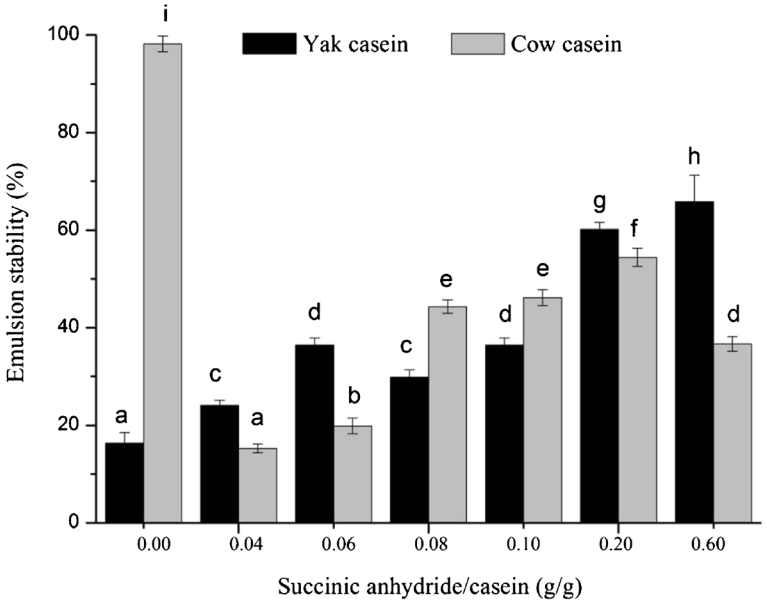

Fig. 3 Emulsion stability of succinylated yak and cow caseins. *Columns* with *different letters* are significantly different (n=3, P<0.05)

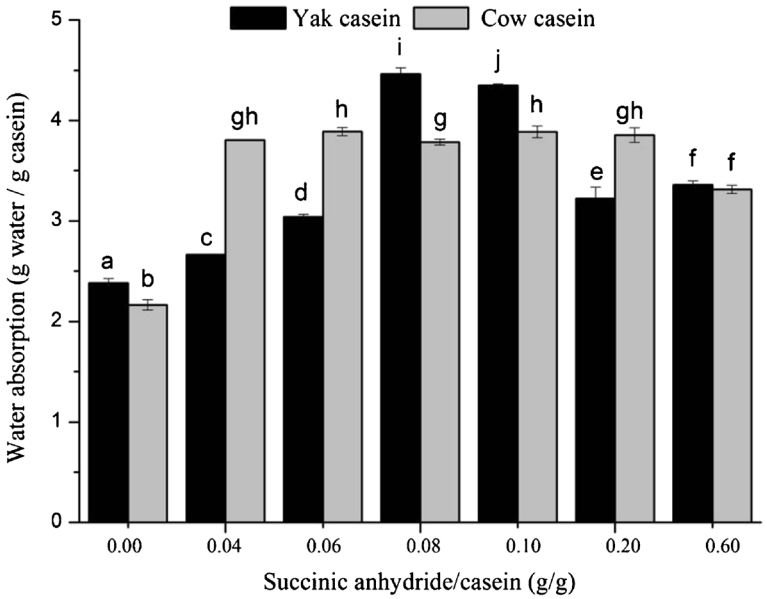

Fig. 4 Water absorption of yak and cow caseins. *Columns* with *different letters* are significantly different ($n=3$, $P<0.05$)

Ma 1999). Compared to cow caseins, an increase in solubility improved the emulsion stability of yak caseins.

3.4 Water and oil binding capacity

The results of water absorption capacity of modified yak and cow caseins are shown in Fig. 4. The water absorption capacity of yak caseins increased at lower succinylation levels and decreased with increasing succinylation, whereas the water absorption capacity of cow caseins increased at lower levels of succinylation and changed slowly with increasing succinylation. Increased water absorption of caseins at lower succinylation levels might be attributed to an increase in protein–water interactions as a result of increased solubility and net charge.

Succinylation results in electrostatic repulsions between the added carboxyl groups and the neighboring native carboxyl groups, resulting in protein unfolding and exposure of buried amino acid groups, making them available for interactions with the aqueous medium (Bora 2002). This result is in accordance with the findings reported by El-Adawy (2000), Mirmoghtadaie et al. (2009), and Lawal and Adebowale (2004). At higher level of succinylation, the reduced water absorption capacity of caseins might be due to changes in conformation, exposure of hydrophobic groups, and reduced casein–water interactions.

Oil absorption capacity of caseins increased after succinylation (Fig. 5). With increasing succinylation, the oil absorption capacity of yak caseins increased gradually and then decreased. In the case of cow caseins, the oil absorption capacity increased gradually except with 0.6 g succinic anhydride. Oil absorption capacity is affected by several factors including protein content, hydrophobicity, surface area, charge and

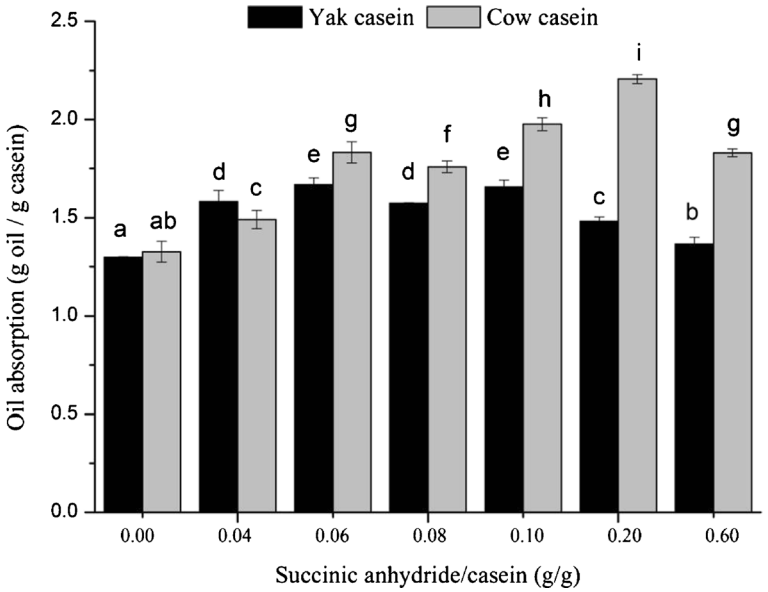

Fig. 5 Oil absorption of yak and cow caseins. *Columns* with *different letters* are significantly different (n=3, P<0.05)

topography, oil liquidity, and method used (El-Adawy 2000). The increase in oil absorption capacity of caseins might be attributed to a reduction in bulk density after succinylation. However, yak caseins micelle has larger size than cow caseins micelle; the dissociation of yak caseins micelle decreased the micellar size at higher succinylation levels, contributing to a reduction in surface area and oil absorption capacity.

3.5 Foaming properties

The effects of succinylation on the foaming capacity of caseins are shown in Fig. 6. Increased yak caseins solubility after succinylation enhanced foaming capacity because soluble proteins contribute to foaming (Chan and Ma 1999). Furthermore, the increase in foaming capacity of yak caseins might be attributed to changes in surface hydrophobicity. It has been reported that a good balance of hydrophilic and hydrophobic groups is necessary for foaming capacity and foam stability (Townsend and Nakai 1983).

Succinylation resulted in a significant reduction of foaming capacity in cow caseins. The balance of hydrophilic and hydrophobic groups of cow caseins might be weakened by succinylation, thereby negatively affecting foaming capacity. Negative charges on proteins prevent the formation of stable foams and decrease foaming capacity after succinylation even though solubility increases foaming capacity (El-Adawy 2000). With increasing succinylation levels, the foaming capacity of cow caseins increased, which was due to increasing solubility and changes in conformation. After succinylation, the foaming capacity of yak caseins was higher than that of cow caseins. The results revealed that succinylation contributed to a good balance of hydrophilic and hydrophobic groups in yak caseins but not in cow caseins.

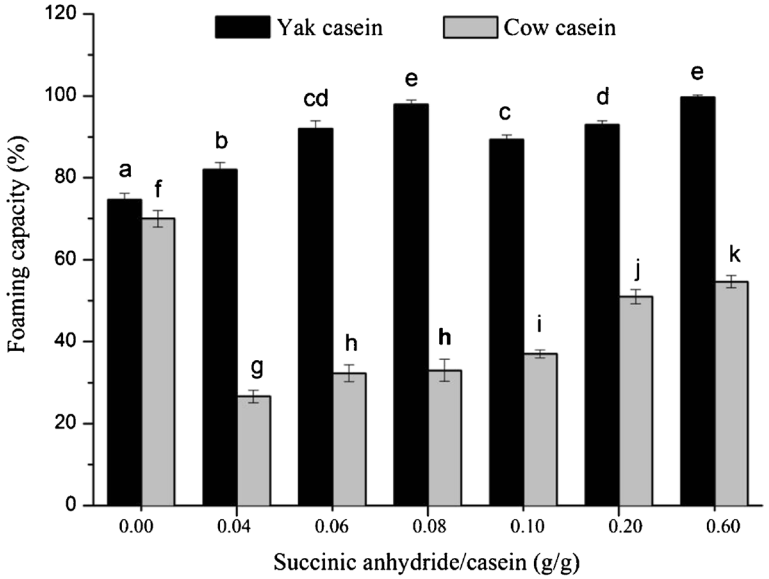

Fig. 6 Foaming capacity of succinylated yak and cow caseins. *Columns* with *different letters* are significantly different (n=3, P<0.05)

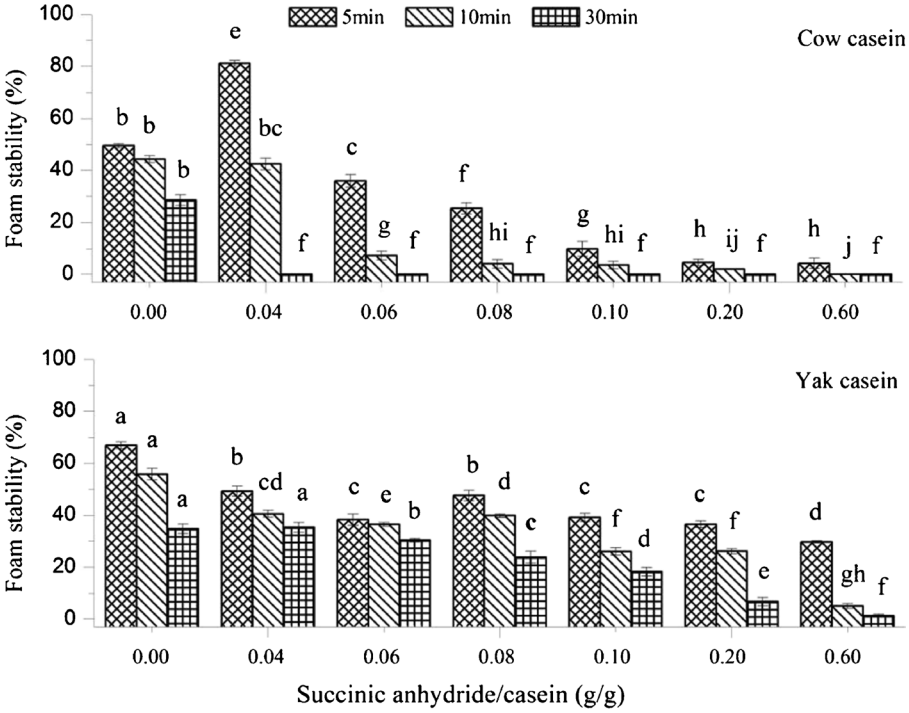

Fig. 7 Foam stability of yak and cow caseins. *Columns* of same time with *different letters* are significantly different (n=3, P<0.05)

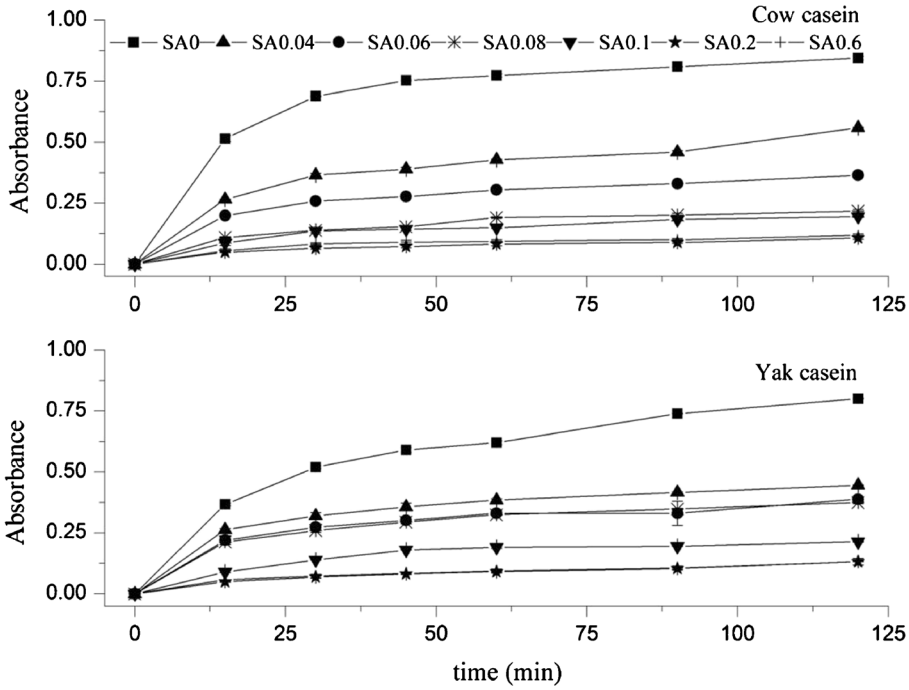

Fig. 8 In vitro digestibility of succinylated yak and cow caseins (n=3)

Foam stability of succinylated caseins decreased (Fig. 7) because excessive increases in charge reduce protein–protein interactions and prevent the formation of an elastic film at the air–liquid interface (Mirmoghtadaie et al. 2009). Cow caseins had lower foam stability than yak caseins with the exception of 0.04 g succinic anhydride, which was attributed to changes in hydrophobicity and conformation. Cow caseins had maximal foam stability with 0.04 g succinic anhydride.

3.6 In vitro trypsin digestibility

Digestibility of succinylated caseins by trypsin is shown in Fig. 8. The digestibility of caseins significantly decreased ($P<0.05$) after succinylation. With increasing succinylation levels, the digestibility of caseins gradually decreased. The rate of TCA-soluble nitrogen formation was high during the first 30 min of digestion and decreased with increasing incubation and succinylation level. At lower succinylation degree, the digestibility of cow caseins was higher than yak caseins, which was due to the better solubility of cow caseins. At high level of succinylation, the digestibility of yak caseins was close to that of cow caseins and even higher, which was attributed to the increase of solubility of yak caseins with succinylation. During succinylation, the association between bulky, negatively charged succinyl groups and lysine residues increased steric hindrance and trypsin–casein repulsions. With increasing succinylation levels, steric hindrance and repulsion increased and digestibility was poor.

4 Conclusion

The effects of succinylation on the functional properties of yak and cow caseins, including solubility, emulsion capacity, water and oil absorption capacity, foaming capacity and digestibility were studied. It was showed that succinylated levels of yak and cow caseins were different with similar succinic anhydride addition. After succinylation, the solubility, emulsifying activity, water/oil absorption capacity of both yak and cow caseins were enhanced, while foam stability and digestibility were reduced. Succinylation decreased both foaming capacity and emulsion stability of cow caseins, and increased that of yak caseins. Succinylated yak caseins had higher functional properties than its native counterpart except for foam stability and digestibility. With increasing succinylation levels, the functional properties of caseins were affected to different degrees. It was concluded that succinylation had different effect on different caseins resources because of the differences on its conformation and composition. The digestibility of modified caseins will be studied in the future in order to observe the consequences of succinylation on susceptibility to other proteases.

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