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Actinidin-induced hydrolysis of milk proteins: Effect on antigenicity

S. Kaur [a], T. Huppertz [a,b,c], T. Vasiljevic [a,*]

[a] Advanced Food Systems Research Unit, Institute for Sustainable Industries & Liveable Cities and College of Health and Biomedicine, Victoria University, Melbourne, VIC, 8001, Australia
[b] FrieslandCampina, Amersfoort, the Netherlands
[c] Food Quality & Design Group, Wageningen University & Research, Wageningen, the Netherlands

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ABSTRACT

Actinidin was used to hydrolyse proteins in whey protein isolate (WPI) and milk protein concentrate (MPC) to reduce immunoreactivity of β-lactoglobulin (β-LG) and αs_1-casein (αs_1-CN). Samples were incubated at an enzyme to substrate ratio of 1:100 (5.21 units of actinidin activity g^{-1} of protein) at 10 or 60 °C for up to 31 or 5 h, respectively. Protein hydrolysis was determined by the degree of hydrolysis and SDS-PAGE. Antigenicity of hydrolysates was determined by β-LG and αs_1-CN antibody-binding capacity using enzyme-linked immunosorbent assay (ELISA) quantification kits. ELISA showed significant reduction of antigenicity of β-LG and αs_1-CN with higher degree of hydrolysis (DH) by actinidin. At 60 °C, hydrolysis for 5 h resulted in antigenicity reduction of ~43% for β-LG and ~48% for αs_1-CN in MPC and ~54% for β-LG in WPI. Hydrolysis at 10 °C for 31 h also resulted in decrease in antigenicity in MPC for β-LG and αs_1-CN by ~39 and 42% respectively, but only 14% for β-LG in WPI. Treatment with actinidin can reduce the antigenicity by modification of protein conformation and cleavage and masking of epitopes of β-LG and αs_1-CN.

1. Introduction

Actinidin (EC 3.4.22.14) is a thiol plant protease obtained from kiwifruit with a molecular weight of 23.5 kDa and contains 220 amino acids (Boland & Singh, 2013). The enzyme was first described by Arcus (1959) but, thus far, actinidin has been sporadically assessed for the applications in the dairy industry. Recently, we evaluated the performance of actinidin on selected milk protein ingredients (Kaur, Huppertz, & Vasiljevic, 2021), noting that the enzyme hydrolysed preferentially whey proteins, especially α-lactalbumin (α-LA). The activity was also temperature-dependant with the greatest degree of hydrolysis (%DH) of ~16% being obtained at 60 °C after 5 h. The enzyme lost activity above 65 °C (Kaur et al., 2021). This indicated that actinidin could potentially be used for modifications of dairy proteins, e.g., for improvements of certain functional properties or minimizing antigenicity.

The allergenic nature of milk proteins can result in some limitations to its application in some foods (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Cow milk protein allergy (CMPA) is the most prevalent food allergy among infants (2–6%) in Western countries and is both IgE and IgG mediated (Bartuzi, Cocco, Muraro, & Nowak-Węgrzyn, 2017; Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). The allergenic nature of milk proteins, such as the caseins (CN), β-lactoglobulin (β-LG), bovine serum albumin (BSA) and α-lactalbumin (α-LA), is due to the presence of conformational and sequential epitopes. Milk proteins exert their allergenicity/antigenicity by binding with IgE and/or IgG, which ultimately may induce an allergy reaction in humans (Bogahawaththa, Buckow, Chandrapala, & Vasiljevic, 2018).

While avoidance of foods containing milk proteins has been one approach in the management of CMPA, this is not a recommended practice due to importance of these proteins as well as other dairy components, e.g., minerals and other micronutrients, in the diet. Therefore, establishing approaches to diminish allergenicity of these proteins is of great importance. Molecular structure of milk proteins can be modified by several approaches, including heat treatment (Bogahawaththa et al., 2017), fermentation (Jia et al., 2021), pressurization, non-enzymatic glycosylation (Bu, Luo, Chen, Liu, & Zhu, 2013) and enzymes (Wróblewska & Troszyńska, 2005). Many studies have shown that caseins, unlike whey proteins, maintain their allergenicity even after heat treatment (Castillo & Cassola, 2017; Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2004. Extensively (eHF) or partially hydrolysed (pHF) infant formula are recommended as first alternative in CMPA children. Differences exist between these formulas, especially in

* Corresponding author.
E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

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the amount of β-lactoglobulin since its level in pHF is substantially greater than that in eHF (Exl & Fritsche, 2001). Modern hydrolysed infant formulas differ due to protein source, a degree and type of hydrolysis and profiles of released peptides, all of which are enzyme dependant in addition to other pre- and post-processing methods (Exl & Fritsche, 2001). The main focus of research activities in this area has been placed at diminishing allergenicity of proteins while retaining other properties most notably sensory acceptability. While numerous studies have been conducted and knowledge in this area has been expanded, the research is still ongoing on improving these formulas (Davisse-Paturet et al., 2019; Virtanen et al., 2021). Thus, further studies are needed with a multitude of objectives including enzyme selection and establishment of processing conditions that would result in hypoallergenic hydrolysates. While some plant proteases, including papain, were recently assessed for reduction of cow milk antigenicity (Liang et al., 2020), actinidin has not been evaluated so far.

The focus of this study thus was to examine the effect of actinidin-induced protein hydrolysis on the antigenicity of αs_1-CN and β-LG. The focus was on β-LG and α_{S1}-CN, since more than 50% of human population is sensitised mainly by these proteins (Rezvan Asghar, Ahmad, & Reihane, 2018). β-LG and αs_1-CN appear to be the main allergens (Schulmeister et al., 2009; Wal, 2004) and studies conducted on a large group of infants showed the allergenic nature of cow milk protein was due to mainly these two fractions (Schulmeister et al., 2009; Wal, 2004). αs_1-CN is a single chain phosphoprotein, comprised of 199 amino acids, and has the highest potential for allergenicity due to presence at least seven epitopes (Cerecedo et al., 2008; Cong, Yi, Qing, & Li, 2013). β-LG is the most abundant whey protein with 162 amino acid residues and molecular weight of ~18.3 kDa. The structure of β-LG has 9 strands, A to I, with the first two described as the main regions that possess allergenic potential (Maier, Okun, Pittner, & Lindner, 2006). Since allergenic properties of these proteins can be reduced by hydrolysis into peptides and free amino acids (Hajihashemi, Nasirpour, Scher, & Desobry, 2014), we hypothethised that using actinidin under commercially relevant conditions may achieve appreciable antigenicity reduction. As allergenicity can be due to two types of epitopes, the research question was whether conformational changes could be related to changes in the antigenicity. In this work, two commercially important dairy products - milk protein concentrate (MPC) and whey protein isolate (WPI) in solutions – were hydrolysed by actinidin at two temperatures (10 and 60 °C) and its effect on antigenicity of αs_1-CN and β-LG was studied.

2. Materials and methods

2.1. Materials

WPI (90%, w/w, protein) and MPC (80%, w/w, protein) were obtained from Fonterra Co-operative (Palmerston North, New Zealand). Actinidin, with an activity of 521 units/g dry matter, based on the CBZ method (Heinrickson & Kézdy, 1976), was kindly donated by kiwiEnzyme.com Ltd (Martinborough, New Zealand). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich Pvt Ltd (Castle Hill, NSW, Australia) and Merck KGa (Darmstadt, Germany). A bovine β-LG enzyme-linked immunosorbent assay (ELISA) quantification kit was obtained from Bethyl Laboratories Inc. (Montgomery, TX, USA) and the bovine αs_1-CN ELISA quantification kit was purchased from SAB (College Park, MD, USA).

2.2. Sample preparation and enzymatic hydrolysis

Dispersions (5%, w/w) of WPI and MPC in simulated milk ultrafiltrate (SMUF) were prepared as described previously (Kaur et al., 2021). Hydrolysis of WPI and MPC was performed with actinidin at the enzyme to substrate ratio (E:S) of 1:100 (5.21 units of actinidin activity/g of protein) at uncontrolled pH at 60 °C for 0–5 h and at 10 °C for 0–31 h in a shaking water bath followed by enzyme inactivation and sample storage for further analysis as described previously (Kaur et al., 2021). This specific temperature (60 °C) was chosen as the degree of hydrolysis (% DH) was the greatest, at ~16% after 5 h at this temperature (Kaur et al., 2021). The enzyme appeared to be denatured above 65 °C. Aliquot samples (1.0 mL) were taken out during hydrolysis at above mentioned time intervals and then 1.0 mL of 5% (w/v) SDS was added followed by heating at 80 °C for 5 min to terminate enzymatic reaction. Such treated samples were stored at −20 °C for further analysis. For antigenicity testing, 1.0 mL of a 5% SDS solution at 80 °C was added to 1.0 mL of sample to stop enzymatic activity. The original samples without added enzyme were treated under the same conditions and served as controls.

2.3. Analytical methods

2.3.1. Determination of the degree of hydrolysis

The degree of hydrolysis (%DH) was determined using the method of Adler-Nissen (1979) by determination of free amino groups using the trinitrobenzenesulfonic acid (TNBS) procedure as described previously (Kaur et al., 2021). %DH was calculated on a basis of the complete hydrolysis (Kaur et al., 2021) as:

$$\%DH = \frac{h}{h_{tot}} * 100 \quad (1)$$

where h_{tot} is the total number of peptide bonds per protein equivalent obtained by given samples with chemical hydrolysis, and h is the number of hydrolysed bonds following enzymatic hydrolysis.

2.3.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analysed by SDS-PAGE under reducing conditions using β-mercaptoethanol, as described by Bogahawaththa et al. (2017), to monitor hydrolysis of individual milk proteins. The gels were scanned using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, Hercules, CA, USA). The gel quantification was achieved by a software (Image Lab 6.0.1 @2017, Bio-Rad Laboratories Inc). All determinations were performed in triplicate.

2.3.3. Fourier transform infrared (FTIR) spectroscopy

Immediately after sampling, all samples, including controls, were analysed using a FTIR spectrometer (Frontier, PerkinElmer, Boston, MA, USA). After using SMUF for a baseline correction, the samples were scanned in the 4000–600 cm^{-1} range with 16 scans per spectra at a resolution of 4 cm^{-1}. The spectra were resolved with Origin Pro 2020 software (Origin Lab Corporation, Northampton, MA, USA). First, the peak deconvolution was performed using the Fourier self-deconvolution (FSD) function within broad amid I region of 1600–1700 cm^{-1}. Baseline correction was performed followed by peak finding and peak fitting with aid of the Gaussian function with required level of iterations. The following four structural motifs were assessed for changes: β-sheets (1637-1610 cm^{-1} and 1696-1680 cm^{-1}), random coils (1648-1638 cm^{-1}), α-helices (1660-1650 cm^{-1}), and β-turns (1679-1667 $cm^{-1)}$ (Grewal, Huppertz, & Vasiljevic, 2018).

2.3.4. Determination of antigenicity of β-LG and αs_1-CN

Samples were assessed for antigenicity using the bovine β-LG (for MPC and WPI) and αs_1-CN (for MPC only) ELISA quantitation kits according to the manufacturers' instructions, as previously described (Bogahawaththa, Chandrapala, & Vasiljevic, 2019; Kleber, Krause, Illgner, & Hinrichs, 2004; Rahaman, Vasiljevic, & Ramchandran, 2017). In brief, 96-well microtiter plates were coated with capture antibody (polyclonal rabbit IgG) that were raised against native bovine β-LG. On the other hand, bovine αs_1-CN detection kit was received with an already coated plate.

Samples and standards were serial diluted to maintain β-LG and αs_1-CN concentration limits within ranges of 1.95–125 ng/ml and 0.78–50

ng/ml, respectively. Exactly 100 μL of standards and diluted samples were added to a well separately and incubated for 1 and 2 h at room temperature and 37 °C for β-LG and αs_1-CN, respectively. After addition of β-LG-detecting antibody and tetramethylbenzidine (TMB) substrate, (or Detection Reagent A followed by Detection Reagent B in case of αs_1-CN substrate solution, the plates were kept in the dark for 15 min for colour development. The reaction was stopped by adding sulphuric acid and the absorbance was measured at 450 nm using a microplate reader (iMark, Bio-Rad Laboratories, Gladesville, NSW, Australia). A standard curve was constructed with each standard absorbance against its corresponding concentration (ng/ml). From the curve, the antigenicity (mg/ml) of β-LG and αs_1-CN was quantified. The proportion (%) of residual antigenicity (RA%) was expressive in relation to appropriate control sample:

$$Residual\ antigenicity\ (\%) = \frac{Antigenicity\ of\ hydrolysate}{Antigenicity\ of\ control\ sample} \cdot 100\% \quad (2)$$

2.4. Statistical analysis

All experiments were replicated 3 times on separate occasions for both substrates and the data were expressed as the mean ± SD of three independent assays. The data was analysed as repeated in time measurements using a SAS software (v. 9.1) and the GLM protocol. The means were compared using the LSMEANS function with the PDIFF option and significance level was set at $p < 0.05$. Several parameters were correlated including antigenicity reduction with residual intact β-LG or residual intact α_{s1}-CN, antigenicity with FTIR and correlation of residual intact proteins (SDS-PAGE summed scores based on proteins composition) with DH. These parameters were analysed by Pearson's correlation coefficient (r) that measures the degree of association between the variables using the IBM® SPSS® statistics software (student v.).

3. Results

3.1. Impact of temperature on hydrolysis and its correlation with proteolytic pattern and secondary structure of proteins

In line with previously reported data (Kaur et al., 2021), temperature had a substantial effect on the extent of protein hydrolysis by actinidin, with more extensive hydrolysis, determined as %DH (Fig. 1) and SDS-PAGE (Table 1 and Fig. 2) observed after hydrolysis at 60 °C than at 10 °C. The %DH obtained at 10 °C appeared in line with those reported

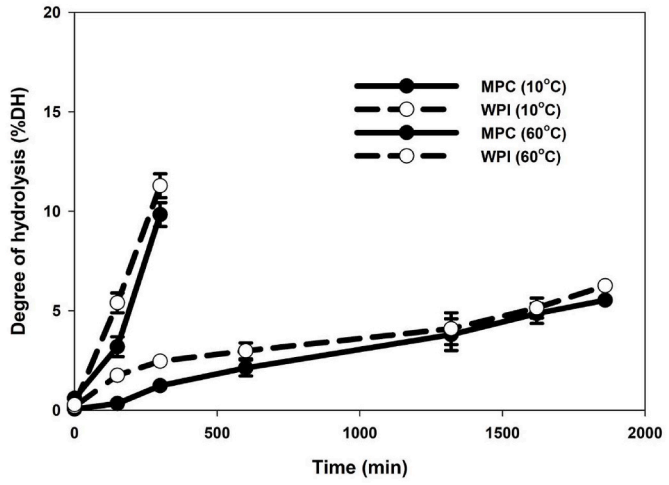

Fig. 1. Degree of hydrolysis (%DH) of 5% MPC and WPI solutions after incubation with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

Table 1
Proportion (%) of intact milk proteins remaining (relative to control) after incubation of 5% MPC and WPI solutions with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

Protein	MPC/10 °C				MPC/60 °C		
	Time of hydrolysis (h)						
	0.16	2.5	10	31	0.16	2.5	5
αs_2-CN	63.2^{aF}	55.0^{bD}	27.1^{eF}	19.5^{gF}	51.9^{cF}	47.6^{dC}	21.1^{fE}
αs_1-CN	74.1^{cC}	65.1^{dC}	35.7^{fC}	32.7^{gA}	94.8^{aA}	77.3^{bA}	53.9^{eA}
β-CN	65.6^{aE}	45.0^{bG}	31.1^{eD}	24.0^{gC}	38.2^{cG}	33.7^{dE}	30.4^{fB}
κ-CN	65.9^{aE}	48.1^{cE}	17.7^{eG}	10.4^{fG}	62.7^{bC}	28.8^{dG}	17.6^{eF}
β-LG	73.7^{bD}	46.2^{dF}	30.4^{eE}	22.8^{gE}	88.8^{aB}	59.5^{cB}	28.6^{fC}
α-LA	55.8^{aG}	0.0^{cH}	0.0^{cH}	0.0^{cH}	38.7^{bG}	0.0^{cH}	0.0^{cG}
	WPI/10 °C				WPI/60 °C		
	0.16	2.5	10	31	0.16	2.5	5
β-LG	84.2^{aB}	84.1^{aA}	46.4^{cA}	23.8^{fD}	60.9^{bD}	40.7^{dD}	27.8^{eD}
α-LA	92.0^{aA}	74.7^{bB}	42.8^{dB}	27.6^{fB}	58.9^{cE}	31.7^{eF}	21.1^{gE}

The pooled standard error of the mean (SEM) of at least 3 independent observations for hydrolysis of MPC and WPI and was 1.32 and 0.28, respectively; lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

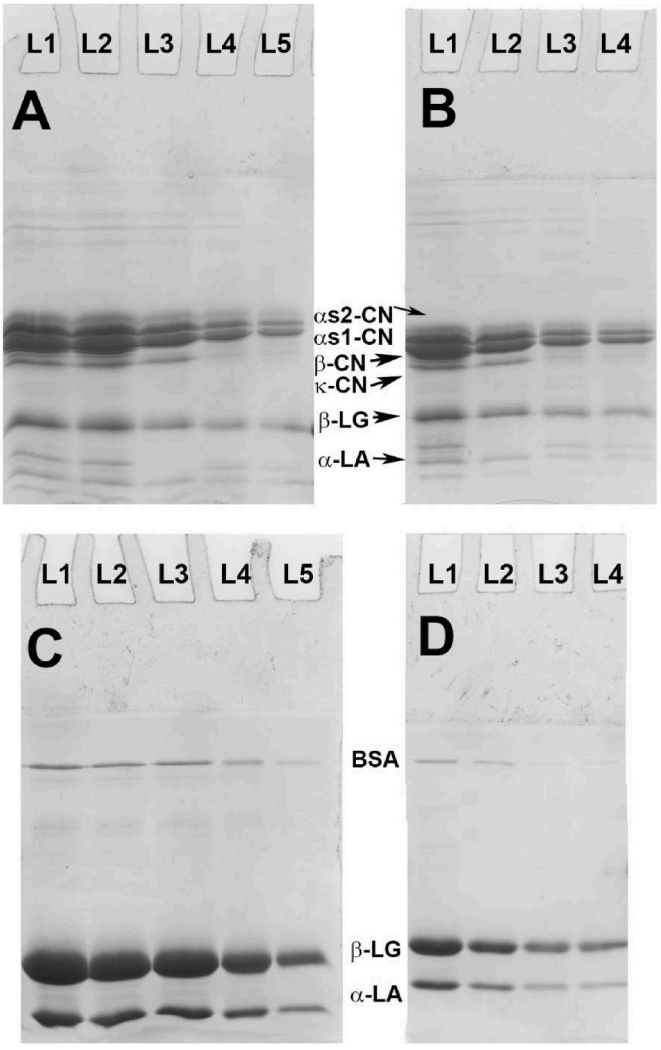

Fig. 2. Reducing SDS- PAGE patterns of MPC (A,B) and WPI (C,D) after incubation with actinidin at a 1:100 enzyme to substrate ratio at 10 °C (A,C) or 60 °C (B,D). L 1 = Control; 2 = 0.16 h; 3 = 2.5 h; 4 = 10 h; 5 = 31 h.

by Liang et al. (2020), who used papain to hydrolyse cow's milk at 20 °C for 2 h. The increase in %DH after 5 h at 60 °C was approximately two times higher than that after 31 h at 10 °C for both MPC and WPI (Fig. 1). Interestingly, however, the proportion of residual intact milk proteins did not show this trend (Table 1). In fact, residual intact proteins were higher after 5 h at 60 °C than after 31 h at 10 °C for all proteins except α-LA in MPC, and were higher for β-Lg but lower for α-LA in WPI (Table 1). The difference between hydrolysis monitored by %DH and SDS-PAGE is that the former considers every peptide bond hydrolysed in a protein, whereas the latter is based on the first peptide bond hydrolysed in a protein. The higher %DH at 60 °C than at 10 °C (Fig. 1) thus suggests more extensive hydrolysis of (poly)peptides from proteins that had undergone initial hydrolysis, rather than more protein molecules being subjected to hydrolysis. This indicates that the initial hydrolysis of the intact protein, rather than the subsequent further hydrolysis of the formed peptides, is rate-determining.

In addition to %DH and SDS-PAGE, protein hydrolysis was also monitored by FTIR (Table A1). In general, though, only limited changes were observed in structural elements of the milk proteins and clear trends as a function of incubation time, temperature and protein source were not readily apparent (Table A1). This could be linked to fact that % DH was comparatively low (Fig. 1) and that although the majority of the proteins had been hydrolysed at end of the incubation at both temperatures (Table 1), the initial peptide bonds hydrolysed in a protein are typically those readily accessible and not those involved in structural elements in the proteins (Buczek, Krowarsch, & Otlewski, 2002; Siezen & Hoenders, 1979).

3.2. Influence of actinidin hydrolysis on antigenicity in MPC and WPI

In addition to protein hydrolysis, changes in β-LG and α_{S1}-CN antigenicity were studied, the results of which are shown in Table 2. Reductions in antigenicity of β-LG were observed in both MPC and WPI and for MPC reductions in α_{s1}-CN were observed, the extent of which increased with increasing incubation time. For β-Lg in MPC, both incubation for 5 h at 60 °C and 31 h at 10 °C resulted in a decrease in antigenicity by ~40%, whereas in WPI, a notably larger decrease in β-Lg antigenicity after 5 h at 60 °C (>50% reduction) compared to 31 h at 10 °C (<15% reduction) despite both having a comparable level of residual intact β-Lg (Table 1). It is also worth noting that the reduction in residual intact β-Lg was larger than that in residual β-Lg antigenicity. In other words, the hydrolysis of a single peptide bond in β-Lg is insufficient to reduce antigenicity. For α_{S1}-CN in MPC, for both incubation at 10 and 60 °C comparable levels of residual intact α_{S1}-CN (Table 1) and residual α_{S1}-CN antigenicity (Table 2) was found, with again reductions in intact α_{S1}-CN being larger than those in α_{s1}-CN antigenicity.

4. Discussion

The allergenic nature of milk proteins limits their use in food products for some parts of the population. Allergenicity is due to the presence of specific amino acid sequences in the primary and secondary structures of proteins/peptides. Epitopes can be linear, conformational or overlapping, but conformational epitopes appear the cause of most allergies (Cong et al., 2013). Proteolysis can disrupt linear and conformational epitopes (Bu et al., 2013) and is one of the approaches to reduce antigenicity of milk allergens (Alting, Meijer, & van Beresteijn, 1998). Furthermore, proteolysis may also lead to formation of new epitopes or appearance of hidden epitopes (Bu et al., 2013). In this study, actinidin, as an unexplored plant protease, was assessed for its impact on milk proteins. Our previous study (Kaur et al., 2021) showed that actinidin can act over a broad range of temperatures, thus the extremes (10 and 60 °C) were applied in the current study. Antigenicity could be related to the content of intact proteins as a highly positive relationship was observed (Table A2). Hydrolysis of substrates containing α_{S1}-CN and β-Lg fractions by actinidin led to a significant reduction in antigenicity of these milk proteins. The ability of actinidin to reduce immunoreactivity of MPs by its hydrolytic action was temperature and time dependent and a correlation could be clearly observed.

Substantial differences between β-Lg and α_{S1}-CN in binding patterns of IgG antibodies to epitopes reflect their differences in the structure, where β-Lg was structurally more stable and compact due to possession of two disulphide bonds in contrast to α_{S1}-CN which is regarded as flexible and unstructured protein (Bu et al., 2013). Actinidin mostly cleaves amino acids present on hydrophobic sites of proteins such as Leu, Val or Phe (Boland & Singh, 2013). For αs_1-CN, the epitopes that could contain a possible cleavage sites include f(21–35) and f(161–175) (Cong et al., 2013). For β-Lg, the main allergenic epitopes include f (41–60), f(102–124) and f(149–162) (Bogahawaththa et al., 2017), all of which contain at least one of three possible cleavage sites. Cleavage of these epitopes may lead to a reduction in the antigenicity. These epitopes are also part of defined structural elements of β-Lg; for example, f (41–60) is part of β-strands and present at the surface of the molecule (Fox, 2003), therefore it was expected to see some structural changes upon their cleavage, which was not clearly demonstrated by the FTIR analysis. To further elaborate on the specificity, using a well-defined system could be considered.

A substantial antigenicity reduction was obtained even at the low temperature (Table 2), and a positive correlation was cursorily observed between remaining antigenicity and residual intact α_{S1}-CN or β-Lg (Table A2) which could likely be attributed to the effect of temperature. α_{s1}-CN has only a small amount of a defined secondary structure (α-helix, β-sheets, β-turn) and lacks disulphide bonds, thus its spatial conformation is stabilised by mainly hydrophobic interactions

Table 2
The antigenicity of β-LG and αs_1-CN (mg/ml) after incubation of 5% MPC and WPI solutions with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

β-LG								
Sample	0	Hydrolysis time (h)						
		0.16	2.5	5	10	22	27	31
MPC/10 °C	2.5^{aE}	2.5^{aE}	2.2^{bE}	2.1^{bE}	1.8^{cC}	1.7^{cC}	1.6^{dC}	1.5^{dC}
MPC/60 °C	2.1^{aF}	2.0^{aF}	1.5^{bF}	1.2^{cF}				
WPI/10 °C	14.8^{aC}	14.8^{aC}	14.8^{aA}	14.5^{bA}	13.9^{cA}	12.8^{dA}	12.8^{dA}	12.8^{dA}
WPI/60 °C	17.3^{aA}	15.8^{bB}	11.0^{cC}	8.0^{dC}				
αs_1-CN								
Sample	0	Hydrolysis time (h)						
		0.16	2.5	5	10	22	27	31r
MPC/10 °C	16.8^{aB}	16.5^{bA}	12.2^{cB}	12.0^{dB}	10.7^{eB}	10.5^{fB}	10.5^{fB}	9.9^{gB}
MPC/60 °C	12.9^{aD}	10.3^{bD}	7.8^{cD}	6.7^{dD}				

The pooled standard error of the mean (SEM) of at least 3 independent observations was 0.036; lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

(Kumosinski, Brown, & Farrell, 1991). Since hydrophobic interactions are limited at low temperature, that would likely lead to more random structures and changes in conformational epitopes. In addition, low temperature also alters casein interactions and diffusion of individual caseins out of the micelle making them more accessible to the enzyme but also creating rather a more porous micellar structure that could be accessed by the enzyme (Bhat, Dar, & Singh, 2016).

Furthermore, α_{S1}-CN antigenicity reduction was greater in the early stages of proteolysis similar to observations obtained in another study (Dąbrowska et al., 2020), in which hydrolysis of α_{S1}-CN by *Yarrowia lipolytica* for 1 h resulted in antigenicity reduction by $\leq$ 30%. Further increase in %DH during 5 h hydrolysis resulted in a negligible reduction in α_{S1}-CN antigenicity (Dąbrowska et al., 2020). Results from the SDS-PAGE (Fig. 2, Table 1) obtained in our study show that α_{S1}-CN band intensity diminished with the extension of hydrolysis time at low temperature, which could also be related to decline in antigenicity. Also, a high positive correlation coefficient was noted when residual α_{S1}-CN was correlated with the remaining antigenicity, even at low temperature (Table A2).

At high temperature, α_{S1}-CN antigenicity reduction was much greater, which could again be related to the extent of hydrolysis. Furthermore, it is clearly evident from the SDS PAGE gels (Fig. 2, Table 1) that αs_1-CN was very susceptible to hydrolysis at this temperature. The similar trend was reported previously when rise in temperature resulted in a greater degree of hydrolysis and greater reduction of antigenicity of milk proteins by latex peptidase (Oliveira et al., 2019).

Results of WPI hydrolysis at 60 °C also concur with the levels of β-Lg antigenicity, where the degree of change in antigenicity of β-LG was significantly greater (~46% antigenicity reduction) as compared to that whey protein hydrolysates obtained at 10 °C (Table 2). This could be related to the extent of hydrolysis as WPI hydrolysis at 60 °C showed highest %DH among all samples. In addition, shifting of the peaks in the FTIR interferograms has been observed (Table A1) indicating changes in the secondary structure that may have disrupted conformational epitopes and led to a slight reduction in antigenicity at low temperature. Due to very low increase in %DH, these changes were potentially more due to impact of temperature and to a lesser extent enzymatic action.

5. Conclusion

Actinidin was able to substantially reduce the antigenicity of αs_1-CN and β-Lg in a time and temperature dependent manner. Both substrates underwent greater hydrolysis at 60 °C than that at 10 °C with a strong negative correlation between %DH and antigenicity. Greater %DH resulted in greater reduction of antigenicity, likely due to cleavage of conformational epitopes. While actinidin was effective in hydrolysing αs_1-CN at both temperatures, this protease was not as effective during hydrolysis of β-Lg at low temperature. On the other hand, greatest %DH of β-Lg was achieved at 60 °C leading to significant antigenicity reduction. These results indicate that milk protein hydrolysates obtained by actinidin had reduced levels of antigenicity due to modifications of protein conformation or cleavage and masking of conformational and linear epitopes of the tested antigens. It could potentially be used in applications where reduction of allergenicity is required.

CRediT authorship contribution statement

S. Kaur: Conceived the study, Research question, Designed, Wrote the, Writing – original draft, Conceptualization, Writing – review & editing, Designed the tables, The figures, Methodology, Formal analysis, Investigation. **T. Huppertz:** Provided critical feedback, Formal analysis, Secured, Funding acquisition, Writing – review & editing, Supervised the study. **T. Vasiljevic:** Provided critical feedback, Formal analysis, Secured, Funding acquisition, Writing – review & editing, Supervised the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113294.

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