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STUDY OF THERMAL AND HYDROLYTIC DENATURATION OF CASEIN (αS_1) USING DIFFERENTIAL SCANNING CALORIMETRY (DSC)

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ABSTRACT

Among various casein variants, α-S_1 has been identified as potential allergen for the sensitive individuals. In this study various thermal and hydrolytic techniques have been employed to denature casein for its extended use as functional ingredient especially to manage allergy. This study aims to denature casein (αS_1) for characterization as functional ingredient in various food and nutraceutical formulations. Casein was denatured using standard thermal (dry, wet and microwave heat) and hydrolytic (using various enzymes) techniques. Denatured casein was characterized using Differential Scanning calorimetry (DSC). Native casein showed thermal transition (ΔH_d) at 0.079 J/g corresponding to the denaturation temperature 103.757^0C. Denatured casein showed new epitopes according to various degrees of denaturation as reflected by variable enthalpies (ΔH_d) corresponding to specific denaturation temperatures. Dry heat treatment for 60 minutes and wet for 45 minutes showed significant thermal denaturation of casein giving 11.05 and 193.10 J/g enthalpies corresponding to 109.775 and 122.472 ^{0}C and 0.002 and 0.002 J/g enthalpies corresponding to 116.425 and 120.173 ^{0}C denaturation temperatures respectively. Similarly, hydrolytic denaturation of casein by various enzymes showed variable enthalpies (ΔH_d). Casein showed new epitope formation due to which it can be used as diversified functional ingredient.

Key words: Casein, hydrolysis, thermal denaturation, DSC.

INTRODUCTION

Casein, main component of milk protein is of four types αs_1, αs_2, β and k-casein with a ratio of αs_1:αs_2 and β:k as 4:1 and 4:1 respectively (Fox 1989; Elzoghby *et al.* 2011). Casein is widely used to elucidate various aspects of protein chemistry (Fox 2003). It is also commonly employed as functional ingredient in diversified food, pharmaceutical and nutraceutical formulations (Bryant and McClements 1998; Dalgleish 1997). Casein micelle is a fragile and dynamic structure which can be represented better by an ensemble of interconverting states than by an average state. The content of an essential amino acid has remained at a low level probably because higher levels would be incompatible with the unfolded conformation needed for caseins to form a thermodynamically stable complex with amorphous calcium phosphate (Holt. 2016). Among caseins, αS_1 casein appears to be the most common allergens for milk-allergic individuals especially its C-terminal, the most hydrophilic region which has been identified as a common binding site for IgE (Docena *et al*. 1996; Swaisgood. 1992; Pihlanto and Korhonen. 2003). The five genetic variants of bovine αs_1 casein results from deletions and single amino acid substitutions at the C- and N- terminal regions of the molecule (Swaisgood. 1992). Identified antioxidative peptides of casein range from 2-14 amino acids in length containing hydrophobic amino acids at the N-terminus and/or C-terminus and proline, histidine, or tyrosine within the sequence (Li and Li. 2013).

Instead of treating the clinical symptoms of allergy, an alternative approach is to modify the allergenic proteins so that they no longer elicit a response. Various food processing conditions can modify the structure of dairy proteins which might alter their immunodominant epitopes thereby modulating allergenicity (Besler *et al*. 2001). The resultant effects are likely to be process dependent. Theoretically, allergy can decrease because of the destruction of epitopes or it can increase because of the formation of new epitopes or an improved accessibility of cryptic or hidden epitopes after allergen denaturation (El-Agamy. 2007; Sathe *et al.* 2005; Heyman. 1999). Moreover, milk protein peptides have quite diversified functions as Food and Drug Administration has approved over 60 peptides as therapeutics (Fosgerau and Hoffmann. 2015). Most bioactive peptides are derived from major milk proteins including significant proportion from αS_1 casein. Identified bioactive peptides can easily be modified to improve activity as analogues of milk protein-derived bioactive peptides have been investigated (McClean *et al*. 2014). The αS_1 casein is also a big source of Antimicrobial Peptides (AMPs) and little homology has been revealed between human and cow αS_1 casein. Certain Opioid peptides that bind opiate receptors and

exert opiate like effects have also been identified from αS_1 casein (Nielsen *et al*. 2017).

Among various approaches to denature casein, heat treatment and enzymatic hydrolyses are very likely to impact allergy. Food allergens are generally resistant to extremes of heat by resisting denaturation and degradation which allows the allergen to survive the digestive system long enough to interact with the immune system to either sensitize the individual or to trigger a reaction. Effects of temperature cannot be attributed to changes in charge. More likely, such efforts are attributable to a weakening of hydrophobic interactions at lower temperature, thereby weakening the interactions of caseins in the caseinate particle (Farrell *et al*. 2013) and allowing some swelling of the particles (Huppertz *et al*. 2017). Enzymatic hydrolysis to degrade milk proteins is widespread within the food industry with the purpose to reduce allergy thereby obtaining nutritional substitutes for milk allergic children (Host and Halken. 2004). Porous casein micelle structure is in line with the apparently free diffusion of various compounds like various enzymes in and out of the micelle (Huppertz *et al*. 2017). Considerable research has been carried out on the functional behaviour of protein hydrolysates (Groleau *et al*. 2003; Ju *et al*. 1995; Liu *et al*. 2001; Otte *et al.* 1997).

In the peptide self-assembling process, hydrophobic interaction, hydrogen bonding, salt bridges, p–p interaction and cation–p interaction all play important roles. A variety of hydrolyzed milk formulae based on caseins with different degrees of hydrolysis (partial or extensive) are commercially available. The antigenicity of those formulae is profoundly reduced (Chirico *et al*. 1997). But, even if the majority of the extensively hydrolyzed formulae developed for milk allergic children are well tolerated, their consumption is known to have triggered allergic reactions in several cases (Wroblewska *et al*. 2004). Even then enzymatic hydrolysis is the most effective and common method for reducing the danger posed by food allergens.

During denaturation processes, rupture of hydrogen bonds may lead to endothermic reactions, and disruption of hydrophobic bonds may lead to exothermic reactions. Such enthalpy changes can be detected by differential scanning calorimetry (DSC) (Raikos. 2010). DSC is used to investigate heat-induced conformational or structural changes of a broad range of food ingredients in various physico-chemical conditions (Dean *et al*. 2001; Privalov. 1992).

In current study DSC has been employed to study denaturation of α-casein. From the calorimetric experiments, the temperature dependence of the partial heat capacity of a protein can be obtained. Analysis of this function could yield fundamental parameters of a thermally and hydrolytically induced transitions like transition temperatures and enthalpies of casein denaturation. Finding of this study can help developing more understanding to engage casein as diversified functional ingredient.

MATERIALS AND METHODS

Materials: Lyophilized bovine αS1 casein (≥ 70%) was purchased from sigma Aldrich Co. USA). All chemical reagents used were of analytical grade (J.T. Baker Chemical Co., Phillipsburg, NJ, USA; Fisher Chemicals, Colonnade, ON, Canada and Anachemia, Lachine, QC, Canada).

Thermal treatments; Dry heat treatment was applied at the rate of 140^0C by putting the sample in the oven for 20, 30 and 60 minutes. For the wet heat treatment, casein sample was put in a plastic tube which was dipped into the water bath at 90^0C for 30, 45 and 60 minutes intervals. Microwave heat treatment was applied to the casein sample under the power of 7 in a conventional microwave oven for 1, 3 and 5 minutes.

Enzymatic hydrolysis of casein (α-S_1): The following procedures were employed for the enzymatic hydrolysis of casein (α-S_1). Control solutions was prepared under the same conditions but without addition of enzyme. All experiments were performed in triplicate.

Pepsin hydrolysis: Casein (α-S_1) (10 mg) was solubilized in 1mL 0.1M HCl. The solution was then incubated at 37°C and the pH was adjusted to pH 2 using NaOH 1M. The hydrolysis reaction was started by the addition of a pepsin (10 mg/mL) at an enzyme: substrate (E:S) ratio of 1:20. The solution was gently stirred for 6h, and then the reaction was stopped by heating at 80 °C for 15 min. The pH was then be adjusted to 7.0.

Chymotryptic and tryptic hydrolysis: The protein (10 mg) was solubilized in 1mL of 0.1M sodium phosphate buffer, pH 8.0. The solution was incubated at 37 °C and the hydrolysis reaction was started by the addition of a chymotrypsin or trypsin solution (10 mg/mL) at an E:S ratio of 1:100. The solution was gently stirred for 4 h, and then the reaction was stopped by heating at 80 °C for 15 min. The pH was then adjusted to pH 7.0.

Simulated gastrointestinal hydrolysis:

Preparation of glycine-HCl solution: A 50 mL 0.2M glycine solution was added to 200 mL flask then 44 mL of 0.2M HCl was added and the volume was raised to the mark on neck by adding water. The pH of the solution was adjusted to the value of 2 by using 2M HCl drops.

Protein hydrolysis: Casein (αs_1) was hydrolyzed first by pepsin subsequently adding trypsin and chymotrypsin enzymes. A 5% w/w of protein sample was added to glycine-HCl solution. The enzymes were added in enzyme/substrate ratio of 1/250 (w/w). The pH was

regulated by Titralab (TIM 865, Radiometer Analytical SAS, Villeurbanne, France). The pH was maintained at 2 for digestion by pepsin for two hours. Following the addition of trypsin and chymotrypsin, pH was maintained at 6.5 for 2.5 hrs. During the total period of hydrolysis the temperature of 37^0C was maintained with constant agitation. The enzymatic reaction was stopped by reducing the pH to 4. The hydrolysate was frozen and proceeded to freeze drying and stored afterwards for further analysis.

Flavorzyme hydrolysis: To 100 mL of 10% protein powder/substrate in a sterile screw-cap bottle, 0.1% enzyme was added and incubated at 60^0C overnight (20 hrs) in a shaking incubator and deactivated the enzyme at 70^0C for 30 minutes. The solution was freeze dried and stored at -86^0C temperature for further evaluation.

Differential Scanning Calorimetry (DSC): Thermal properties of the casein samples were studied by DSC. Each sample (15 µL) of protein solutions (15%) were hermetically sealed in aluminium DSC pans and heated. A scan rate of 5^0 C/min was used and samples were heated under helium through the range of 20–140^0 C. An empty pan was used as reference. Calorimetric measurements were carried out using DSC-Q 200 (TA Instruments, Inc., New Castle, USA) and data was analyzed using a Universal Analysis TA Software. The instrument was calibrated using indium as standard.

RESULTS AND DISCUSSION

Thermal denaturation of casein: DSC results of various heat treatments of casein are presented in Table 1 and Fig. 1-3. The denaturation temperature (T_d) and enthalpy change (ΔH_d) were determined from the maximal peak temperature and the area of the peak, respectively. Native casein showed thermal transition (ΔH_d) at 0.079 J/g corresponding to the denaturation temperature 103.757^0C. At neutral pH, αS_1 casein associates to polymers. The unfolded conformation of casein exposes side chain groups buried in the native structure, and the perturbation process causes an increased reactivity of thiol groups (Paulsson and Dejmek. 1990).

Thermal denaturation of protein involves the disruption of intramolecular bonding and the unfolding and aggregation of protein molecules. Due to the application of various kinds of heat treatments, differences in enthalpies and denaturation temperatures occur. Dry heat treatment to casein for 20 and 30 minutes time showed 9.537 and 0.619 J/g enthalpies corresponding to 128.065 and 137.477^0C denaturation temperatures respectively. Dry heat treatment to casein for 60 minutes time showed 11.05 and 193.10 J/g enthalpies corresponding to 109.775 and 122.472^0C denaturation temperatures respectively. Similarly, the wet heat treatment for 30 minutes showed 0.227 J/g enthalpy corresponding to 82.279^0C denaturation temperature. Wet heat treatment for 45 minutes showed 0.002 and 0.002 J/g enthalpies corresponding to 116.425 and 120.173^0C denaturation temperatures respectively. Wet heat treatment for 60 minutes showed enthalpy 502.00 J/g corresponding to 74.653^0C denaturation temperature. For the microwave heat treatments for 1, 3 and 5 minutes exposure, the enthalpies were 1504.00, 0.021 and 1505.00 J/g corresponding to the denaturation temperatures of 103.607, 118.430 and 112.040 ^{0}C respectively. These results fall within the range 25-165 °C temperature corresponding to an enthalpy of 154.34 J/g of the DSC analysis of casein which indicated an endothermic process (Moldoveanu *et al.* 2013). Furthermore, unfolding of the protein results in an increase in free volume and molecular mobility, and this requires heat to occur. Transitions that involve a process (denaturation and gelatinization) shift to higher temperatures at higher heating rates. This is due to the fact that amount of time at a given temperature decreases as heating rate increases (Thomas and Schmidt. 2010).

Moldoveanu *et al*. (2013) have noticed two degradation domains of thermal denaturation of casein, an endothermic (two stages) and the exothermic (three stages). The casein is thermally stable between 0-172 °C. First degradation stage develops between 41.9°C - 172.4°C. These low temperatures are indicative of the elimination of the physically retained water in casein (4.60%). The thermal stability of casein is expressed by the initial degradation temperature of the second stage, Ti = 172.4°C (Moldoveanu *et al.* 2013). Aggregation of casein micelles is probably caused by restructuring of the micelles induced by changes of the pH and the temperature. Restructuring leads to a progressive change of the balance between repulsive and attractive interaction between the micelles with increasing temperature in favor of the latter and drives aggregation when it reaches a critical value at a critical temperature. Hydrophobic interactions, hydrogen bonding and complex formation between calcium, phosphate and phosphoserines are all involved in the gelation process (Thomar and Nicolai. 2016).

Moreover, results of the current study are in line with the Thermogravimetric, Differential Scanning Calorimetric (TG-DSC) analysis of casein variants where the weight loss occurred between 30-130^0C and it was found that intercalated proteins degrade at relatively higher temperatures (Yu *et al*. 2010).

Table 1. Denaturation temperatures and enthalpies of casein at various heat treatments studied by differential scanning calorimeter (DSC).

Variant	Denaturation temperature (^{0}C)	Denaturation enthalpy (J/g)
Casein (native)	103.757	0.079
D-20 min	128.065	9.537
D-30 min	137.477	0.619
D-60 min	109.775	11.05
	122.472	193.10
W-30 min	82.279	0.227
W-45 min	116.425	0.002
	120.173	0.002
W-60 min	74.653	502.00
MW-1 min	103.607	1504.00
MW-3 min	118.430	0.021
MW-5 min	112.040	1505.00

Legend: D = Dry; W = Wet; MW =Microwave; min = Minutes

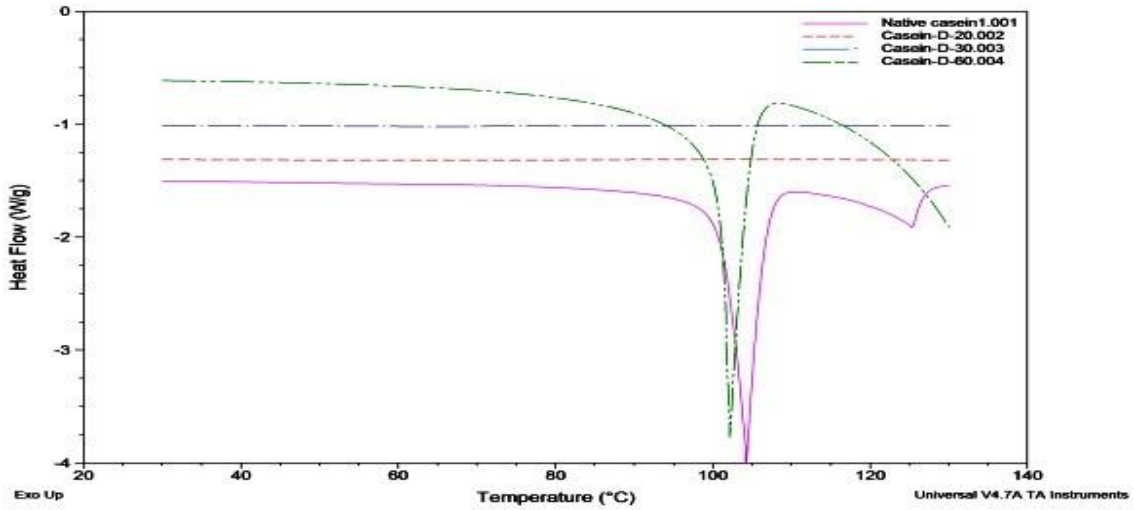

Figure 1. Thermograms of dry (D) heat treatments applied to casein α-S$_1$ at 140^{0}C for various time intervals (D-20, 30 & 60 minutes) studied using differential scanning calorimetry (DSC).

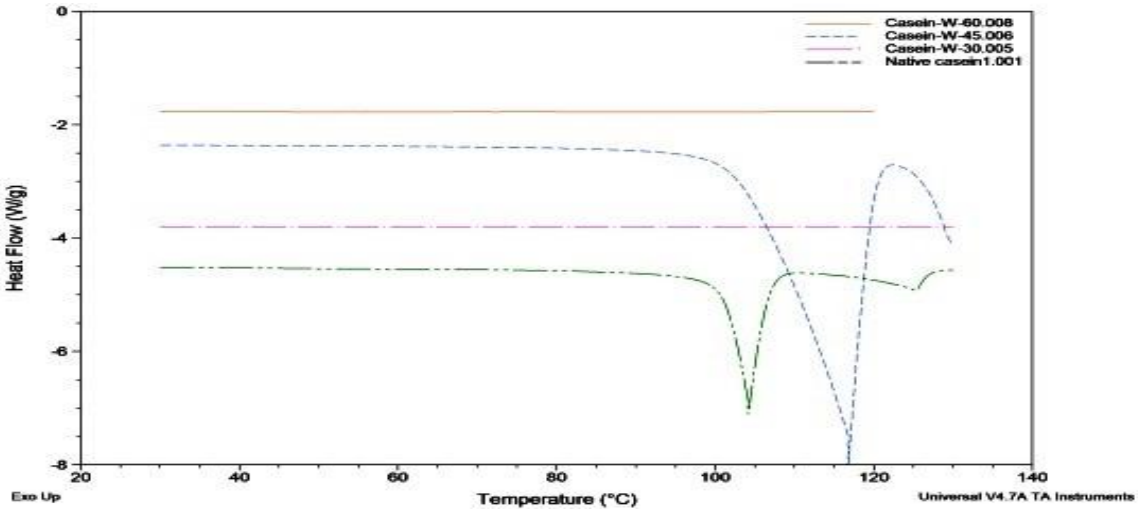

Figure 2. Thermograms of wet (W) heat treatments applied to casein α-S$_1$ at 90^{0}C for various time intervals (W-30, 45 & 60 minutes) studied using differential scanning calorimetry (DSC).

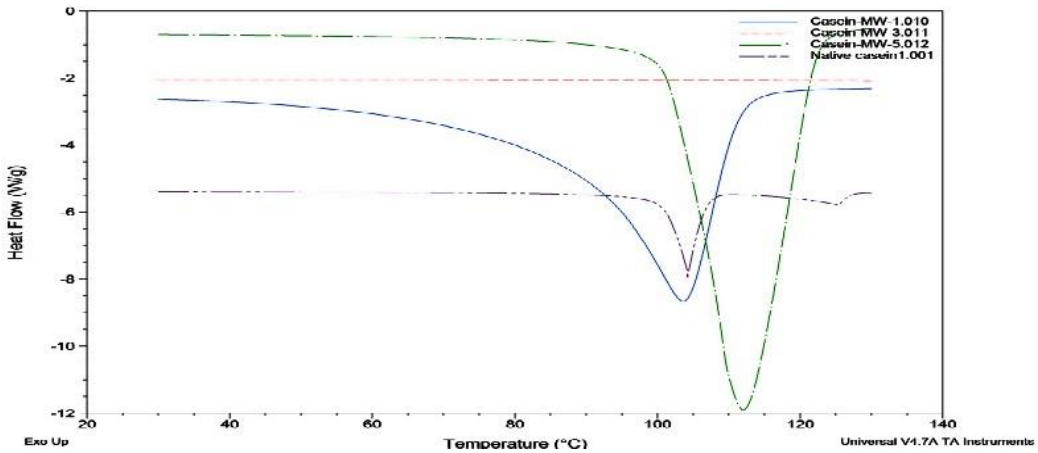

Figure 3.Thermograms of microwave (MW) heat treatments applied to casein α-S_1 at power of 7 for various time intervals (MW-1, 3 & 5 minutes) studied using differential scanning calorimetry (DSC).

Hydrolytic denaturation of casein: When the casein is hydrolysed/ digested, it is degraded into an array of peptide fragments that have different biological effects as compared to the native/parent protein. The peptides of α-S_1casein have been reported to have an array of activities like antimicrobial, angiotensin-converting enzyme (ACE) inhibition, dipeptidyl peptidase IV (DPP-IV) inhibition, opioid agonist and antagonist activities, immunomodulation, mineral binding and antioxidative functions (Nielsen *et al*. 2017). The resultant peptides are absorbed into the blood stream and function systematically as ACE-inhibitory peptides have the potential to lower blood pressure. The fragments of αS_1 casein have been detected in the blood of an adult human for a period of 8 hours after consumption of milk or yogurt (Chabance *et al*. 1998). DSC results of various enzymatic hydrolysates of casein are presented in Table 2 and Fig. 4. Extensive intraandinter-subunit disulfide bonds provide greater stability against thermal denaturation. Therefore, enzymatic hydrolyses provide significant differences in the thermal transition behaviour of casein.

Table 2. Denaturation temperatures and enthalpies of casein for its various enzymatic hydrolysates studied by differential scanning calorimeter (DSC).

Variant/hydrolysate	Denaturation temperature (^{0}C)	Denaturation enthalpy (J/g)
Casein (native)	103.757	0.079
Pepsin	50.518	816.20
	108.768	144.40
Trypsin	84.115	1324.00
	117.967	120.50
Chymotrypsin	88.518	1374.0
	123.550	176.20
Gastrointestinal	105.187	1219.00
Flavorzyme	109.802	32.18
	134.330	5.170

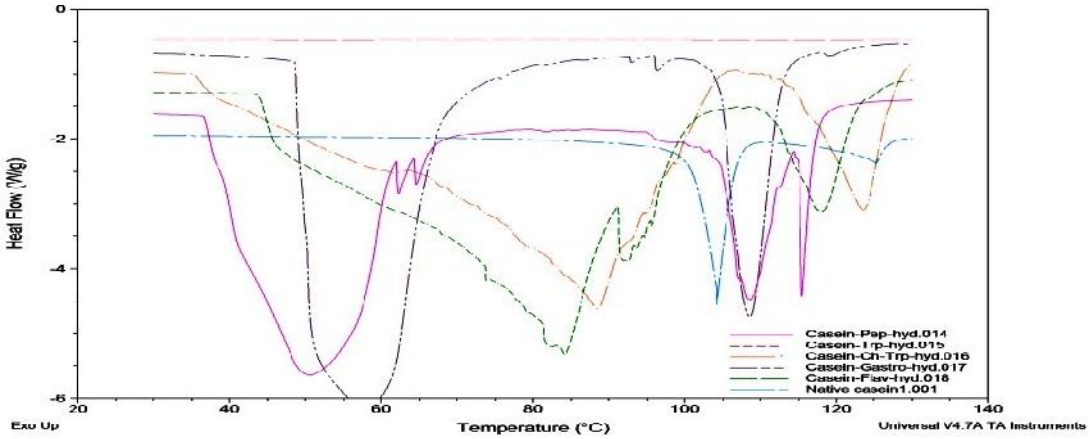

Figure 4. Thermograms of enzymatic hydrolysates (hyd) of casein αS_1 studied using differential scanning calorimetry (DSC)

Legend: Pep = Pepsin; Trp = Trypsin; Ch-Trp= Chimotrypsin; Gastro = Gastrointestinal; Flav = Flavorzyme.

Hydrolysis with the pepsin showed enthalpies 816.20 and 144.40 J/g corresponding to 50.518 and 108.768 ^{0}C, respectively. Similarly hydrolysis of casein with trypsin enzyme showed 1324.00 and 120.50 J/g enthalpies corresponding to 84.115 and 117.967 ^{0}C denaturation temperatures respectively. The hydrolysate of chymotrypsin showed 1374.0 and 176.20 J/g enthalpies corresponding to 88.518 and 123.550 ^{0}C denaturation temperatures respectively. The hydrolysate from simulated gastrointestinal hydrolysis showed thermal transition (ΔH_d) at 1219.00 J/g corresponding to the denaturation temperature 105.187^{0}C. The hydrolysate from flavorzyme showed thermal transition (ΔH_d) at 32.18 and 5.170 J/g corresponding to the denaturation temperature 109.802 and 134.330 ^{0}C, respectively. Variation in thermal transitions might be due to variation in degree of hydrolysis as presence of proline can increase peptide resistance to hydrolysis by digestive enzymes (Yang and Russel. 1992). Low hydrolytic activity could be related to high number of proline residues which are likely to survive of a longer period of time in the digestive system and more likely exert in vivo bioactivity (Nielsen *et al*. 2017). The results of the current study are further supported by the findings of Nielsen *et al*. 2017, wherein αS_1 casein has been reported to have high peptide density regions with full or partial peptide sequences. The peptides range from 2-14 amino acids in length where half of the unique sequences have a proline at the second position from the N-terminus, indicating a possible structural pattern for DPP-IV-inhibitory peptides.

The increased entropy of amino acid residues in the unfolded state is balanced by the decreased entropy of water clusters induced by apolar groups. Classically and historically, the intermolecular forces prevailing in protein interactions are ionic +/- bonds, hydrogen bonds and Van der Waals forces. The latter should properly be termed dispersion forces and would include electrostatic- and dipole- or induced dipole-dipole interactions, thereby encompassing the cation-π and π-πforces involved more recently in protein-protein interaction (Waters. 2002 and Dalkas *et al*. 2014). The stabilizing effect of disulfide bridges causes restriction of the mobility of the unfolded peptide backbone (Paulsson and Dejmek. 1990). Ionic bonds are simple +/- interactions between oppositely charged residues such as lysine, arginine or histidine (all+) with glutamate or aspartate (both -) and require no further explanation. Hydrogen bonds are more complex and there is still debate as to their exact nature and the extent to which they possess an electrostatic or covalent character (Hubbard *et al*. 2010 and Martin and Derewenda. 1999). Casein enzyme hydrolysate (CEH) aggregates differ markedly and may represent viable vehicles to sequester hydrophobic insoluble organic molecules (Liu and Guo. 2008). Moreover, multiple clusters of hydrophobic residues are identified as potential reaction sites in the hydrophobic tails and trains of the casein proteins (Horne. 2017). Only one hydrophobic essential amino acid with a low disorder propensity, Leucine occurs at moderate frequency in αS_1 casein. Threonine in αS_1 casein occur less commonly on average than in the other orthologues of bovine casein (Holt. 2016).

Conclusion: Application of various heat treatments denatured casein (αS_1) at various degrees. Enzymatic hydrolysis caused epitope formation at various points. Denatured and hydrolyzed molecule of casein produced various enthalpies of denaturation corresponding to different thermal and hydrolytic treatments. Dry heat for 60 minutes and wet heat for 45 minutes produced two points of enthalpies of denaturation against respective temperatures while rest of each heat treatment produced only at one point enthalpy of denaturation. Likewise, all the enzymes hydrolyzed casein well wherein each enzyme produced two points of enthalpies of denaturation against their respective temperatures except gastrointestinal enzyme which produced only one point of denaturation.

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