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Scientific Background of Dairy Protein Digestibility: A Review

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Recent advances have shown that differences in compositional, structural and physical properties of caseins and whey proteins affect their digestion and absorption behavior, hormonal response, satiety effect and other physiological effects. For example, the ingestion of whey protein cause fast, high and transient increase of amino acids 'fast protein', whereas casein induce slower, lower and prolonged increase of 'slow protein' in the gut. Knowledge of, and control over, the rate and nature of digestive breakdown of dairy proteins provides a potential basis for product/process innovation through identifying ingredients and formulations that provide desired nutrient delivery profiles. With this background, the aim of our current review paper is to understand the digestion behavior of various protein-rich milk powders and their potential use in formulation of dairy foods for controlled release of amino acids and energy. Currently available in vitro protein digestibility methods to measure or predict the dairy protein digestibility were also investigated. The author has also presented the preliminary results of ongoing study on in vitro digestion of various commercial proteins powders.

Keywords: *In vitro* digestion, Caseins, Whey proteins, Processing

Introduction

Proteins are the most important ingredient present in milk and milk products due to its nutritional significance, role in stability and rheology, allergenicity, biological activities of its bio-peptides and various functional properties. Recent advances in dairy sciences have shown the differences in compositional, structural and physical properties of caseins and whey proteins affect their digestion and absorption behavior in the human gut. There is a keen interest in understanding how protein is digested in the gastrointestinal tract as it has been linked to various physiological conditions. Knowledge of, and control over, the rate and nature of digestive breakdown of dairy proteins provides a potential basis for product/process innovation through identifying ingredients and formulations that provide desired nutrient delivery profiles. For example, designing formulations that lead to sustained but complete uptake of proteins for maximal physiological and/or satiety benefits.

Recent studies have shown the ingestion of whey protein cause fast, high and transient increase of amino acids 'fast protein', whereas casein induce slower, lower and prolonged increase of 'slow protein' in the gut (Boirie *et al*., 1997; Dangin *et al*., 2001; Lacroix *et al*., 2006). Casein clotting in stomach thought to cause slow emptying whereas more soluble whey proteins rapidly pass through to duodenum causing post-prandial aminoacidemia (Boirie *et al*., 1997). This leads to greater post-prandial amino acid concentration (25-50%) and β-cell response (insulin, C-peptide, pro-insulin, 12-40%) for whey and free amino acid meals compared to caseins (Nilsson *et al*., 2004). Dietary proteins are more satiating per kJ than carbohydrate and lipids (Anderson *et al.*, 2004). It has been reported that more than 20 peptide hormones present in gut such as cholecystikinin, gastric inhibitory peptide, Glucagon like peptides and regulatory hormones such as regulatory hormones insulin, leptin etc., controlled by central nervous system, regulates the food intake and satiety. The rate of protein digestion not only affect the ability of the body to assimilate amino acids (Crittenden *et al*., 2009), impacts on insulin regulation (Tessari *et al*., 2007) that lead to the stimulation of many physiological and metabolic responses known to be involved in food intake regulation (Anderson and Moore, 2004). These events have implication on the design of (dairy) protein/carbohydrate composite products.

Rationale of protein digestibility studies

The nutritive value of a protein is evaluated by the amino acid profiling. But it is the (degree of) protein digestibility which determines primary availability of peptides and amino acids. The digestibility of a food protein and the bioavailability of peptides or amino acids may be obtained by using rat bio-assay (Hsu *et al*., 1977) which is quite cumbersome. Study of postprandial whole protein metabolism requires human subjects, which involves combining oral and intravenous administration of labeled and unlabelled proteins to measure amino acid kinetics after meal which is an expensive and highly tedious exercise (Boirie *et al*., 1997; Lacroix *et al*., 2006). *In vitro* digestion that mimics the *in vivo* condition is an option for such studies but it has to be backed up by animal or human digestion studies for full validation. The unique passage of foods into the digestive tract where it is exposed to a series of enzymes, varying rheological conditions, constant absorption of degraded protein molecules, hormonal responses to proteins/amino acids is difficult to reproduce. A parallel study of the rheology of dairy components needs to be carried out to identify factors likely to impact on passage rate through the upper alimentary canal. The protein digestibility

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assay, particularly pepsin digestibility, has been used to determine the relative stability of a protein to the extremes of pH and pepsin-protease encountered in the mammalian gastric environment. It was used for assessing the nutritional value of protein sources by predicting amino acid bioavailability (Zikakis *et al*., 1977; Marquez and Lajolo, 1981).

Thermal treatment of milk and milk products at various stages of processing and stabilization is known to affect their nutritional properties (Carbonaro *et al*., 1997). Both endogenous thermolabile components of milk and milk products can be used as an indicator of heat damage, particularly whey proteins. *In vitro* digestibility of processed milks (pasteurized, UHT and sterilized) are higher than raw milk (Carbonaro *et al*., 1997) which is in a way conform that heating of milk and milk production induce the gross changes in protein conformation with subsequent proteolytic cleavage of previously inaccessible sites (Lyster, 1979; Finley, 1985).

The major allergenic proteins of peanut, soybean, egg and milk have been determined in a pepsin digestion assay using simulated gastric fluid (SGF). Generally, the allergens and lectins examined in these experiments were resistant to pepsin digestion whereas the other proteins were more rapidly and completely digested. Cow's milk has one of the highest levels of food allergen and is at the top of all lists of epidemiological data. The human gastrointestinal fluids more rapidly digest nutritionally desirable proteins but most of the food allergens exhibit proteolytic stability. β-lactoglubulin is one of such potent milk allergens that show a high stability against proteolytic enzyme. Heat treatment slightly increases the pepsin hydrolysis whereas natural fermentation significantly improve the digestibility of β-lactoglubulin (~40%) (Maier *et al*., 2006). Analysis of cow's milk proteins in infant formula showed the whey proteins, β-lactoglobulin and α-lactalbumen, are entirely resistant to digestion from pH 1.5 to 3, whereas casein showed good digestibility (Sakal *et al*., 2000). A multi-interlaboratory evaluation showed there is no standard protocol for digestibility of proteins measurement with potential variation in pH, pepsin purity, pepsin to target protein ratio, target protein purity and method of detection (Thomas *et al*., 2004).

Maillard reaction of milk proteins is known to occur ubiquitously during processing and storage of milk. It is known to improve many techno-functional properties of the milk products. Hiller *et al*., (2010) showed the post Maillard modification *in vitro* digestion of sodium caseinate decrease digestibility by 36-55% whereas increased in the case of whey proteins. It was concluded that sodium caseinate form tightly weaved networks that sterically hindered the proteolysis. On the other hand, partial unfolding of protein structures during Maillard reaction, relating to hydrothermic conditions may facilitate proteolysis of globular whey proteins. It is also suggested that complex sugars like dextrin leads to unfolding of β-lactoglobulin molecules that making previously inaccessible peptides bond available for enzymatic action.

Literatures on in vitro protein digestibility studies

There have been several studies on *in vitro* methods of protein digestibility. Earlier *in vitro* studies used pepsin-pancreatin (Akeson and Stahmann, 1964), enzyme preparation from *Streptomyces griseus* (Ford and Salter, 1966), papain (Buchanon and Byers, 1969), and papain-trypsin system (Saunder *et al*., 1973) for protein digestibility studies (as referred by Hsu *et al.*, 1977). Saunder *et al*. (1973) found that the values obtained by the enzyme system used by Akeson and Stahmann *et al*., (1964) and Saunder *et al*., (1973) showed excellent correlation with R= 0.88 and 0.91, respectively. However, papain as a sole enzyme (Buchanan and Byers, 1969) showed poor correlation with *in vivo* data. Rhinehart (1975) trials with triple enzyme systems also showed good correlation with *in vivo* studies (as referred by Hsu *et al.*, 1977).

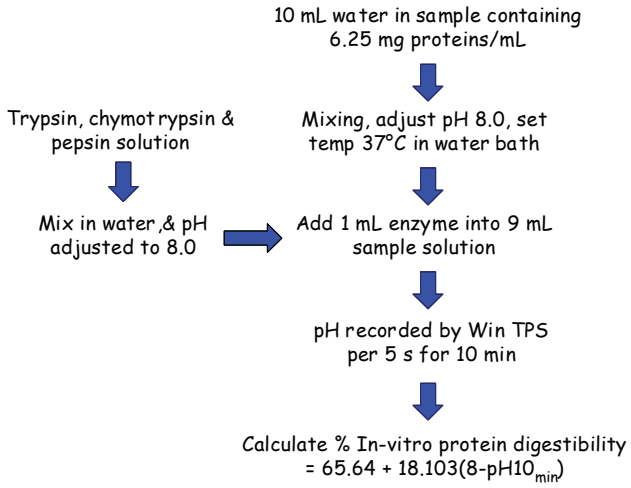

Figure 1. Digestibility protocol for various milk samples

Hsu *et al*., (1977) developed a less complicated, rapid but reliable *in vitro* method where porcine pancreatic trypsin, bovine chymotrypsin and intestinal peptidase were added together to protein samples set at pH 8.0 and drop in pH was measured till 10 min of digestion (Figure 1). On the other hand, they also measured the *in vivo* digestibility of the particular protein sample using rat as a subject. An equation was developed by regression analysis, such as (% digestibility = 210.46 – 18.10 X_1, where X_1 is the pH of the test sample after 10 min). The method appeared to be equally good for various types of foods including dairy products with high degree of correlation, as shown in the Table 1. Digestibility data from the multi-enzyme treatment of 5 different proteins showed the rate of digestion differ greatly. Casein appeared to be undergoing rapid drop in pH, 8.0 to 6.7, as compared to its counterpart whey protein, 8.0 to 7.4, that shows the nature/

composition of proteins dictates their rate of digestibility too (Figure 2). It also indicates casein has faster digestion rate as compared to the whey protein. It is one of the highly cited studies in protein digestibility (429 citations).

Bodwell *et al*., (1980) tested digestibility of six proteins preparation with modified Hsu *et al*. (1979) method (20 min digestion) and 4 enzyme methods (adding bacterial protease). These digestibility data were compared with human and rat assays. In general, the digestibility values obtained from these three *in vitro* studies were not greatly different. For cottage cheese, % digestibilities were 86.5, 92.2 and 87.1% for tri-enzyme, modified tri-enzyme and four enzyme methods, respectively. They concluded the use of *in vitro* enzyme procedures would only give an approximate estimate of digestibility in humans.

Table 1. *In vivo* and *in vitro* measurements of protein digestibility[1]

Samples	*In vivo* digestibility	*In vitro* digestibility	Differences
Casein	90.5	89.2	1.3
Soy isolate	89.6	88.1	1.5
Partially delactosed whey	73.1	76.7	-3.6
Corn-Milo grain	72.0	73.6	-1.6
High protein wheat bran flour	77.5	76.9	0.6
General wheat flour	81.9	85.7	-3.8
Non-fat dry milk	84.7	82.5	2.2
Corn DPC alcohol washed	79.2	79.4	-0.2
Extruded puff A	79.2	81.9	-2.7
Unextruded puff A	78.0	80.3	-2.3
Extruded puff B	85.1	85.0	0.1
Unextruded puff B	82.9	83.6	-0.7
ANRC casein	87.6	88.1	-0.5
Blended C bread	83.0	79.6	3.4
Blend C ingredients	84.4	85.7	-1.3
Wheat protein concentrate	89.9	90.4	-0.5
Yeast protein concentrate	86.5	83.2	3.3
Bean protein concentrate	84.3	84.1	0.2
Soy concentrate	87.7	87.2	0.5
Full lactose whey	78.6	76.5	2.1

[1]Hsu *et al.,* (1977)

Parrot *et al*., (2003) measured the *in vitro* digestibility of cheese (as water soluble extract and casein) using multiple enzymes to study the effect of digestive enzymes on the biological activity of peptides present in dairy products. Initially acidified cheese sample (pH 2) was treated with pepsin and incubated for 30 min and neutralized to stop pepsin activity. Further digestion was performed with trypsin or pancreatin for 4hr, enzyme activaterd and stored at -20°C. The extract was analysed for protein content by SDS-PAGE, N-analysis by Kjeldahl method and peptide analysis by RP-HPLC. Results showed the digestion of cheese extract induce an increase in ACE (angiotensin-I converting enzyme, ACE) inhibition in compared to undigested extract. ACE is a dipeptidyl carboxypeptidase which catalyses both the production of the vasoconstrictor angiotensin-II and the inactivation of the vasodilator bradykinin, the inducing hypertensive effects.

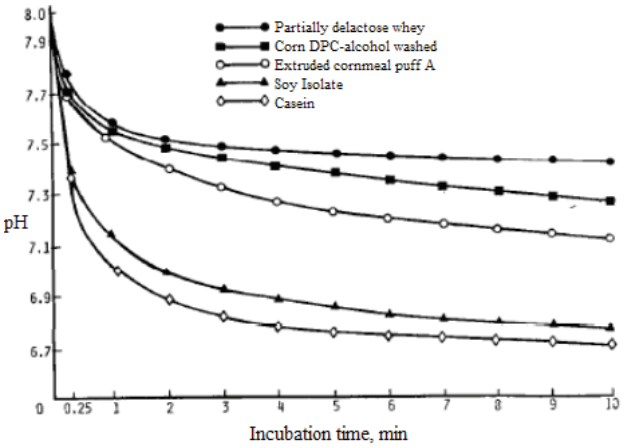

Figure 2. Examples of pH *vs* time curves obtained by incubation of the protein sources with the multi-enzyme systems (From Hsu *et al.,* 1977)

Measurement of peptic digestibility of cow's milk protein in commercially available infant foods under various pH conditions showed interesting results (Sakai *et al*., 2000). Aqueous solutions of infant formula were acidified from 1.5, 2.0, 2.5, 3.0 and 4.0 pH. Dilute NaCl solution was incorporated in one of the solutions (pH 2.0). Digestion of these extracts was done by porcine pepsin at 30, 60 and 120 min at 37°C in reciprocating water bath. The extract were neutralized and subjected to SDS-PAGE and subsequent analysis to determine quantity of protein present. Whey proteins, β-lactoglobulin and α-lactalbumen, were digested at pH 1.5 to 2.5 but almost entirely resistant to peptic digestion at pH 3.0. Casein had similar digestibility from 1.5 to 3.5 pH but slower at pH 4.0. Inclusion of NaCl in did not affect the casein digestibility but lowered digestibility of whey proteins.

In vitro digestion assays have been frequently used to analyze the resistance of pepsinolysis of certain proteins which are potentially allergenic. This has led to a number of *in vitro* studies dedicated to measure the resistance of allergens, whey proteins (particularly β-lactoglobulin), to pepsin hydrolysis (Carbonaro *et al*., 1997; Kitabatake and Kinekawa, 1998; Mouecoucou *et al*., 2004; Maier *et al*., 2006; Roufik *et al*., 2007). The methods used for these vitro studies, however, differed with investigators.

Carbonaro *et al*., (1997) used triple enzyme method of Bodwell *et al*., (1980) to measure %digestibility of 18 variously heat-treated milk samples. They further analyzed the disulfide reactivity and amino acid profiles to come at conclusion that thermal treatment of whey proteins make it less digestible (in heat treated milk and milk products). Maier *et al*., (2006) used peptic digestion of β-lactoglobulin (βLG) containing milk samples and capillary zone electrophoresis (CZE) to study the proteolytic resistance (allergenic proteins). The acidified sample solution (pH 1.5) were incubated with pepsin in 1:20 ratio at 37°C and incubated at different time intervals. Aliquots were taken at different intervals to analyze the proteolytic degradation of β-lactoglobulin level at different time intervals. βLG extract showed minimal digestibility (<2% in 2 h) whereas raw and pasteurized milks were digested up to 45% in 2 h). Mouecoucou *et al*., (2004) studied interactions between βLG and polysaccharides by *in vitro* gastric and pancreatic hydrolysis in dialysis bags followed by measurement of nitrogen release and protein quality by SDS-PAGE. Results showed that βLG was almost resistant to pepsin digestion and the plant hydrocolloids inhibited significantly βLG digestibility as determined by using dialysis bag with a 1000 MW cutoff. Roufik *et al*., (2007) measured the digestibility of antihypertensive peptides from β-lactoglobulin (lactokinins or βLG) and bound complex βLG and β-lactoglobulin variant A (β-LGA) using two step digestion methods similar to Mouecoucou *et al*., (2004) minus dialysis bag. The pepsin digested substrate was withdrawn at various intervals and immediate raised to pH 8.0 and digested with trypsin, chymotrypsin or pancreatin. Reverse Phase HPLC was used to measure the degree of hydrolysis of these individual peptides or their complexes. Digestibility of β-LGA and of the complexes determined using pepsin, trypsin, pancreatin, pepsin/trypsin, and pepsin/pancreatin were similar, whereas chymotrypsin and pepsin/chymotrypsin digested the complexes more slowly.

Dairy protein digestibility-current perspective

Literature survey suggests various factors, from sample preparation to analysis of digesta, might have impact on the digestibility study of milk proteins. For example, allergenicity study of milk protein/peptides warrants digestion of the given protein with pepsin in the simulated stomach condition whereas metabolic study of a given protein may warrant simulated peptic as well as simulated gut conditions. More sophisticated techniques are needed if detail identification of peptides and amino acids are to be analyzed. It is understood that simulation of digestion conditions (as in various parts of alimentary canal) during *in vitro* digestion of proteins is critical. The pH of the substrate (or substrate/enzyme); rheological properties of the mixture and local condition; mixing such as peristalsis, extending/contracting motion, gravity; secretion of several enzymes, bile, mucus etc. can have significant effect on digestion of the protein.

Our previous study on the digestibility of processed as well as granular (raw) starch suggests bulk protein digestibility may be more related to the physical forms such as size of granules, porosity, crystallinity etc. of the ingredient along with its molecular composition (Shrestha *et al.*, 2010; Dhital *et al.*, 2010). For example, there is greater resistance to pepsin digestion for β-lactoglobulin due to numerous disulfide bonds that stabilize structure, but the role of compact globular structure that hinders the access of proteolytic enzymes to the vulnerable protein sites (Carbonaro *et al*., 1997) is equally important. The major protein in the bovine milk, casein, is

well digested because of its poor secondary structure with and more open structure which is vulnerable to proteolysis (Bodwell *et al*., 1980). It is hypothesized that rates and extents of digestion of dairy ingredients (and hence biological effects such as protein accretion and satiety) can be controlled by appropriate selection of raw materials, processing and formulation.

We recently studied the *in vitro* digestion behavior of skim milk powders (SMP) (low heat and medium heat SMP), caseins (casein micelle and sodium caseinate) and whey proteins (concentrate and isolate). These powders were dissolved in water and left at room temperature for few minutes. Each sample solutions were separately heated for 100°C for 1-2 s (Boiling); 66°C for 30 min (pasteurization); 72°C for 15 s (high temperature short time, HTST) and a control (room temperature). These samples were analyzed for protein digestibility characteristics following Hsu *et al.* (1977) (Figure 3). The equation developed by Hsu *et al.*, (1977) as previously described [%Digestibility = 210.46 – 18.10 X_1, where X_1 is the pH of the test sample after 10 min] was used for the calculation of *in vitro* percentage digestibility (IVPD).

The process of protein digestion leads to hydrolysis of polypeptides that eventually lead to formation of smaller peptides and free amino acids. Release of amino acids during digestion is accompanied by a drop in pH of the test solution. The rate of pH drop with time is the function of protein digestibility. Figure 3 showed the skim milk powders had the slowest digestibility trend among the 4 milk ingredients, in all processing conditions. Digestibility curves indicate a mixed result for whey proteins and caseins e.g., casein had faster digestion at room temperature whereas whey proteins had faster digestion during pasteurization (Figure 3). These findings against the common norms that caseins are 'slow digesting' and whey proteins are 'fast digesting'. Two major factors that might have resulted in this opposite trends are: The samples used in the current study were commercially produced. These were exposed to rigorous physical and chemical treatments. Their native structures likely to have significantly altered that potentially affect the enzyme hydrolysis significantly. Carbonaro *et al.*, (1997) also reported that thermal treatment of whey proteins make it less digestible.

In vivo digestion protocol involves exposure of proteins to very low pH (gastric pH ~2.2) and pepsin in stomach. This leads to breakage of the peptide bond between Phenylalanine (at 105) and Methionine (106), micellar flocculation, gel formation and loss of solubility. This event leads to less access of enzymes (trypsin, chymotrypsin and peptidase) to the substrate, slowing digesting significantly. On the other hand, soluble fraction of proteins such as whey proteins are less affected by low pH and the native structure remain more or less unchanged in gastric condition. The smaller whey proteins more likely to be digested by proteolytic enzymes than 'precipitated casein'. Current *in vitro* protocol bypassed the gastric digestion step and only simulates the intestinal digestion. It is most likely to have influenced faster digestion rate of casein as compared to whey proteins. Bodwell *et al.,* (1980) has also previously reported that casein is well digested by proteolytic enzymes because of its poor secondary structure and more open structure.

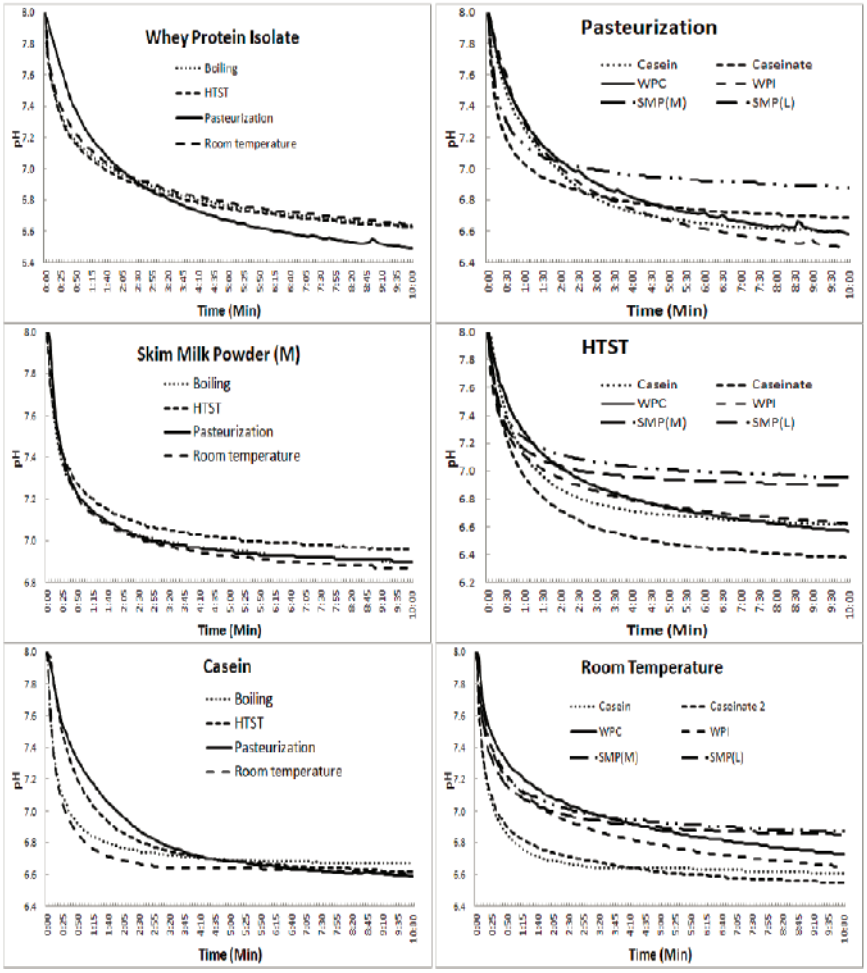

Figure 3. *In vitro* protein digestibilities of various dairy proteins powders under varying processing conditions.

The *in vitro* protein digestibility (IVPD) data (Table 2) also

showed the SMPs have the lowest digestibility as compared to whey proteins and caseins. It appears that previous heat treatment (~85°C), vacuum concentration and spray drying of skimmed milk during manufacturing stages may have altered the protein configuration lowering the protein digestibility. IVPD data also showed that there is a lesser variation in digestibility curves of SMPs under different processing conditions, as compared to others. Table 2 also showed whey proteins had only slightly higher IVPD value as compared to caseins. HTST treatment of sodium caseinate appears to increase IVPD value significantly. It indicates solubilized sodium salts of whey protein tend to be more digestible than casein when boiled. We found the current *in vitro* digestion method is less complicated, short, rapid, and reported to be reliable method of protein digestibility. However, it is not known if this method can differentiate the digestibility behaviour of proteins from various milk sources, as it is very basic mimicking of *in vivo* digestion process and lacking in gastric digestion stage.

For any promising leads, *in vitro* digestion results would need to be backed up by animal or human digestion studies for full validation. The two factors that determine *in vivo* digestion rates are the intrinsic digestion rates (as predictable from *in vitro* studies) and the rate of passage, which is primarily a

Table 2. *In vitro* protein digestibility (IVPD) values of various milk powders

Protein types	%Protein[1]	%In vitro protein digestibility (IVPD)			
		Boiling	HTST	Pasteurization	Room Temperature
SMP-Low heat	33.6	85.8	85.5	84.7	85.5
SMP-Med heat	33.3	85.1	83.9	86.2	86.3
WP Concentrate	76.5	90.3	91.7	91.2	89.6
WP Isolate	90.2	90.6	90.8	92.8	89.9
Casein	86.9	89.8	90.7	89.9	90.5
Na-caseinate	90.2	91.2	94.7	90.0	91.1

[1]Protein analyzed by combustion method used Leco Nitrogen Analyzer

function of rheological properties under the local conditions operating through the digestive tract. There is a transformation from protein in structured food into masticated and bolus formation in the mouth which is suddenly exposed to gastric juice of stomach (pH 1.5 to 3.0) and sudden aggregation. Biosurfactant such as phosphatidylcholine may react with protein to change its structure. In many cases, proteins in food exist as emulsion; in such case proteins/peptides is displaced by phospholipids. Emulsion destabilization takes place in stomach leading to flocculation, coalescence and phase separation. The protein aggregates exposed to shearing or mixing when it exit from pyloric sphincter to the duodenum. In small intestine, protein is exposed to a range of hydrolytic enzymes and range of biosurfactants such as bile acids and phospholipids. Thus a parallel study of the rheology of dairy components would need to be carried out to identify factors likely to impact on passage rate.

Conclusions

Dairy protein is the major source dietary proteins for human kind. The factors affecting the rate of protein digestion from dairy products largely affect the protein utilization and subsequent effect on the human health such as weight management, muscle health, diabetes control etc. Based on digestibility, caseins are classified as 'slow' whereas, whey proteins as 'fast' digesting proteins. Protein molecules are highly sensitive to processing conditions such as heat, acidity/alkalinity, enzymes, pressure etc., that directly or indirectly affect its molecular conformation, digestibility and bioavailability peptides and amino acids. This review showed that digestibility study of the dairy proteins are affected by a number of factors such as type dairy proteins (caseins *vs* whey proteins), physical state of dairy foods, method of analysis e.g., types of enzymes used, steps of digestion, incubation time, etc. and also about various analytic methods to characterize the digested proteins.

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