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Methods of Protein Digestive Stability Assay – State of the Art

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1. Introduction

The evaluation of protein stability in digestive tract is an important topic in various fields of biomedical sciences. The most straightforward task within it is the evaluation of food quality, as during the food processing the digestibility of a protein could fall dramatically. Another task, tightly connected with the previous one, is the evaluation of protein allergenicity. This parameter is shown to be dependent on protein's digestibility, the less digestible ones having the highest probability to induce immune response. Finally, the task of development of new peptide drugs also requires digestive stability check for *per os* drug forms that are the most convenient for a patient.

Current knowledge on protein digestion suggests it to be a multistage process, starting from pepsin cleavage in stomach and proceeding through trypsin and chymotrypsin digestion in intestinal lumen, and finally involving cleavage by intestinal surface and intracellular proteases. The latter two protease groups accomplish the most deep protein degradation. An intriguing point is that in spite of this knowledge the models, which include all stages of protein digestion, are used primarily in food quality control.

Protein digestion, specific enzymes, involved in this process, as well as research area specific methodology for protein digestibility evaluation is regularly reviewed. However, as a common rule, the authors of latter group of reviews ignore the existence of methods with similar goals in other research areas, as well as the question of a degree to which any given method represents a living organism. The aim of this review is to describe existing models for evaluation of protein digestibility with a special emphasis on biological relevance provided by distinct model as well as on productivity of each methodology.

2. Protein and peptide digestion

Before a discussion of protein digestibility evaluation methods, it seems worthwhile to introduce a reader to the current understanding of protein and peptide digestion process in order to provide him with a holistic picture of what could happen to an ingested protein. That is why this section will give a short description of digestive tract and an evaluation of enzymes involved in protein digestion in stomach and intestine. A special emphasis will be laid on characterization of intestinal wall peptidases (surface as well as intracellular) with description of their specificities and their role in overall protein digestion.

2.1 An overview of the protein digestion in the digestive tract

First of all, is should be noted, that excellent reviews and textbooks are readily available on the topic of digestive tract physiology and biochemistry (Freeman & Kim 1978; Widmaier et al. 2011), and we refer a curious reader them for more details; here only a short overview of this process is presented.

The digestive tract consists of several interconnected compartments: mouth (grinding and moisturizing of food), esophagus (connection and separation), stomach, small intestine (food digestion and absorption), and large intestine (food remnants digestion by symbiotic microbiota and undigested material excretion). Protein digestion is initiated in lumen of stomach, then proceeds in the intestinal lumen and is finalized at the surface and in the cytoplasm of intestinal mucosa cells. Additional digestion events occur in large intestine, where symbiotic microorganisms either digest protein remnants to amino acids, or convert them to other compounds of nutritional value.

Stomach is the first compartment, which is chemically active towards proteins. Its acidic pH serves three major functions: it sterilizes the ingested material, denaturates food protein and activates local proteolytic enzymes. Only one protease, pepsin, is secreted in stomach. Pepsin does not destruct proteins to amino acids, rather it splits them to smaller parts to increase its accessibility for successive digestion steps (Widmaier et al. 2011).

The core function of stomach is the control of graduated food delivery into small intestine, and so it is rather dispensable for the overall protein assimilation. The experimental data on animals and patients with total gastrectomy show that the organism even under this condition retains the ability to assimilate dietary protein adequately and remains in positive nitrogen balance (Freeman & Kim 1978).

The next digestion phase, pancreatic, occurs in small intestine. It is thought to be more critical than the gastric phase. Here the pH changes to more basic and several different sources of proteases come into play. The most abundant ones: trypsin, chymotrypsin, elastase and several carboxypeptidases, are secreted by pancreatic acinar cells in a form of precursors, which are activated by proteolytic cleavage (Widmaier et al. 2011). The first three enzymes are endopeptidases; they cleave protein or polypeptide substrates at different specific peptide linkages within their secondary structures. Carboxypeptidases are exopeptidases and act on terminal peptide bonds at the carboxyl terminus of protein. The products of the combined action of the aforementioned enzymes are small peptides and amino acids. These small peptides consist of 2-6 amino acid residues and account for over 60% of luminal amino nitrogen; the remaining 40% is associated with amino acids (Freeman & Kim 1978).

The results of Savoie *et al.* indicate that the amount and size of the resultant peptides and their stability and resistance to exhaustive hydrolysis is strongly influenced by the nature of the source protein. For example, while casein and gluten have the highest proportion of the 1-10 kDa peptide fraction in the first 2 h of digestion, the peptides from gluten are evidently less degraded in the subsequent 4 h. The folding and spatial orientation of the protein chains could explain this observation; the high number of Pro and Glu residues is expected to have contributed heavily in the low accessibility and efficacy of the hydrolytic action of pancreatic enzymes on Pro- and Glu-reach proteins (Savoie et al. 2005).

The core properties of the luminal proteases are summarized in table 1, to give the reader an understanding of the processes, which take place at different stages of digestion. It should be noted that most digestive enzymes are present not as a single entity, but rather as a group of slightly different molecules with quite similar substrate specificity and kinetic properties. Some authors suggest it to be a sign of evolutionary importance of these enzymes (Silk et al. 1976). A detailed discussion of these enzymes is, however, beyond the scope of this chapter, and we refer a curious reader to the excellent reviews available (Freeman & Kim 1978; Turner 1968; Whitcomb & Lowe 2007).

Besides the common luminal enzymes, which are secreted by specialized organ – pancreas, a significant amount of intestinal wall proteases is also present in the lumen. The sources of these enzymes are desquamated mucosal cells resulting from active replacement of the lining epithelium. An approximate quantity of these enzymes is about 30 g per day (Freeman & Kim 1978; Gropper et al. 2009).

Chemical assay data, thermostability studies, and examination of electrophoretic mobilities of luminal peptide hydrolases indicate that aforementioned jejunal enzymes originate predominantly from the cytoplasm of intestinal mucosal cells, whereas the brush border of mucosal cells is a major source of the enzymes in the ileum. All of these enzymes recognize primarily short peptides and are aminopeptidases; they are discussed in more detail in the next section.

The products of luminal proteolysis are free amino acids and small peptides having a chain length of two to six amino acid residues. Analysis of post-prandial intestinal contents aspirated from human jejunum reveals that approximately only one-third of the total amino acid content exists in the free form (Silk et al. 1985). Some of the short peptides are directly absorbed by specific transport systems. However, to increase the overall efficiency of the assimilation process the mucosal cells of intestinal wall provide an additional source of proteolytic enzymes. As these cells line the intestinal wall and are intensively shed into lumen, they contribute both to luminal and parietal digestion. A profound characteristic of mucosal cells is high aminopeptidase activity. This peptidase activity is located in two main subcellular fractions: the cytoplasm and the brush border membrane. The brush border membrane fraction is associated with less than 10% of the total hydrolytic cellular activity for dipeptides but as much as 60% for tripeptides (Freeman & Kim 1978; Sterchi & Woodley 1980a).

The whole digestion process is quite rapid in man, as well as in rat. So, according to the data of Curtis *et al.*, 44.9 % of the meal had left the stomach of a rat in 30 min, and 84.7 % of the meal had left the stomach in 60 min. At 30 min and thereafter, negligible amounts of ^{14}C-labeled protein, which was included in the meal, were detected in intestinal contents of proximal third of small intestine. At 30 min and 1 hr, ^{14}C accumulated in medial and distal thirds of small intestine. At 2, 3 and 4 hr small quantities of ^{14}C were detected in intestinal contents of distal third of small intestine and the colon. The absorption of ingested protein was virtually complete 2 hr after administration of the meal (Curtis et al. 1978). In human, the situation is quite the same: digestion and absorption of dietary protein are completed in the jejunum, the whole process of digestion and absorption reaches its maximum at about 45 min or possibly earlier, and absorption may be largely complete within 75 to 90 min. However, the data of some studies show that the remnants of dietary protein can be recovered from the ileum as late as 4 hr after a meal (Adibi 1976). Nevertheless, it appears

Enzymes	Compartment and localization	Properties	References
Stomach proteases			
Pepsins: Pepsin, pepsin B, gastricsin, chymosin A, renin	Stomach lumen	Endopeptidase, cleaves at Phe, Tyr, Leu, and Val	(Freeman & Kim 1978; Szecsi 1992; Turner 1968; Whitecross et al. 1973)
Pancreatic proteases			
Trypsins: Cationic trypsin, anionic trypsin, mesotrypsin, pancreasin	Ileum lumen	Endopeptidase; cleaves peptide bond at Lys and Arg	(Feinstein et al. 1974; Freeman & Kim 1978; Savoie et al. 2005; Whitcomb & Lowe 2007)
Chimotrypsins: Two isoforms + chymotrypsin-like protease	Ileum lumen	Endopeptidase, cleaves bonds at aliphatic amino acid residues	(Bender & Killheffer 1973; Savoie et al. 2005; Whitcomb & Lowe 2007)
Elastases: Two isoforms	Ileum lumen	Endopeptidase, cleaves at Ala, Gly, Ser	(Whitcomb & Lowe 2007)
Carboxypeptidases A: A1, A2, A3	Ileum lumen	Exopeptidase, cleaves aromatic amino acids from carboxyl terminus of substrate	(Whitcomb & Lowe 2007)
Carboxypeptidases B: B1, B2, N, M, E, AEBP1	Ileum lumen	Exopeptidase, cleaves Arg or Lys from C-terminus of substrate	(Whitcomb & Lowe 2007)
Intestinal wall proteases			
Brush border aminopeptidases (distinct enzymes): aminopeptidase 1, aminopeptidase 2, dipeptidylpeptidase IV, gamma-glutamyltranspeptidase, aminopeptidase A, aminopeptidase M	Small intestine epithelium brush border	Hydrolyze amino acid residue at the N-terminus of hexa- to tripeptides	(Kim et al. 1972; Sterchi & Woodley 1980b)
Cytoplasmic aminopeptidases: four distinct enzymes	Small intestine epithelium cytoplasm	Hydrolyze amino acid residue at the N-terminus of tri- and dipeptides	(Kim et al. 1972; Schiller et al. 1977)

Table 1. Principal properties of protein hydrolases

reasonable to assume that under normal conditions the greatest portion of dietary proteins is digested and absorbed in the upper small intestine (Adibi 1976; Silk 1980; Silk et al. 1985).

2.2 Enzymes of the intestinal wall

In addition to the luminal proteases, the mucosal cells that line the intestinal wall also possess two kinds of proteolytic enzymes: membrane-bound, which are primarily localized in the brush border area, and cytoplasmic. The core difference of these enzymes from the luminal ones is that they are oligopeptidases, that is, recognize only short peptides of 2-6 amino acids in length. The profound characteristics of these enzymes are that they are aminopeptidases, so the N-terminal amino acid residue of peptides is the determinant of their rate and substrate specificity, and all have pH optima in alkaline (7.5-8.5) range (Adibi 1976).

To date, in brush border membranes in human and rat were identified at least 6 aminopeptidases (aminopeptidase 1, aminopeptidase 2, dipeptidylpeptidase IV, gamma-glutamyltranspeptidase, aminopeptidase A and aminopeptidase M) (Kim et al. 1972; Sterchi & Woodley 1980b), and in the cytosol of the mucosal epithelial cells at least 4 enzymes were detected (Kim et al. 1972; Schiller et al. 1977). The specificity towards peptide length is distributed unequally between the subcellular fractions: the majority of dipetides (80 to 95%) are hydrolyzed by cytoplasmic enzymes (Adibi 1976; Krzysik & Adibi 1977), 10-60% of the tripeptidase activity is also in the brush border membrane (Sterchi & Woodley 1980a), and activity against tetrapeptides is located exclusively in the brush border membrane (Sterchi & Woodley 1980a). The ability of brush border enzymes to hydrolyze penta- and hexapeptides has been reported (Adibi 1976), however, little details are still known about this activity.

Some, but not all, cytosolic and brush border membrane peptidases also display some preference to the amino acid composition of substrate. Thus, aminopeptidase 1 from brush border is most active towards aliphatic tripeptides (Sterchi & Woodley 1980b), although most activity against aliphatic peptides is located in cytosol; dipeptidases of brush border prefer substrates with aromatic amino acid at the N-terminus (Sterchi & Woodley 1980a). An interesting exception are proline-containing peptides, which are hydrolyzed by a special hydrolase in the cytosolic fraction (Freeman & Kim 1978; Smith & Bergmann 1944). In normal conditions, 80-90% of hydrolase activity in rat and human is localized within the cytosolic fraction (Kim et al. 1972), starvation increases the peptide hydrolase activity of the cytosol fraction, while that of the brush border fraction is decreased (Adibi 1976).

The distribution of peptide hydrolase activity between brush border membrane and cytosol varies considerably with species (Sterchi & Woodley 1980a), the enzymes from different species have different electrophoretic mobility, but, at the same time, by the activity and substrate preference they often could be grouped in a strikingly similar manner (Kim et al. 1972).

2.3 Peptide and protein transport across the intestinal wall

The absorption is the last and the most controversial phase of protein digestion. Theoretically, an organism requires only amino acids to produce energy and rebuild lost proteins. The foreign proteins and peptides should be avoided, as they could transmit

unwanted signals or even be toxic or mutagenic. The reality, however, is much more complicated. Although amino nitrogen in postprandial blood is almost entirely in the form of free amino acids, few small peptides do appear in portal blood. These include short proline- and hydroxyproline-containing peptides after gelatin ingestion, and both carnosine and anserine after ingestion of certain meats, specifically chicken breast. Significant amounts of whole protein and other large macromolecules are absorbed intact by fetal and neonatal small intestine (Freeman & Kim 1978).

The transport of amino acids is active and depends on a gradient of sodium ions across the brush border membrane of intestinal epithelial cell. Three major group-specific active transport systems exist for: a) monoamino monocarboxylic (neutral) amino acids, b) dibasic amino acids and cysteine, and c) dicarboxylic (acidic) amino acids (Silk et al. 1985). Methionine and the branched chain amino acids (leucine, isoleucine, and valine) are absorbed most rapidly from amino acid mixture. Among the 18 common dietary amino acids, aspartate and glutamate are most slowly absorbed, as are tryptophan and threonine among the eight essential amino acids. As a group, essential amino acids are better absorbed than nonessential amino acids (Adibi 1976).

Small peptides are also actively absorbed, but their transporters are different from those for amino acids. As such, dipeptides and tripeptides are actively transported against a concentration gradient, and they share a common transport mechanism. There are at least two general strategies for transport of small peptides. First, small peptides resistant to brush border membrane peptidases appear to be transported intact across the brush border membrane and are later hydrolyzed by the cytoplasmic peptidases. Second, small peptides, having a higher affinity for brush border membrane peptidases are likely hydrolyzed primarily at the brush border membrane. The hydrolysis products, including free amino acids and dipeptides or tripeptides, are then absorbed by their respective transport systems (Freeman & Kim 1978; Sterchi & Woodley 1980a). There are little data on the active transport of tetrapeptides, and these data do not support the hypothesis on existence of such process (Silk et al. 1985).

One of important differences between amino acid and small peptide transport is that the capacity of free amino acid absorption is far greater in the jejunum than in the ileum, while that for peptide transport is evenly distributed. High-affinity amino acids competitively inhibit the absorption of low-affinity amino acids. However, absorption rates of amino acids by the intestinal epithelial cells are significantly greater in the form of low molecular weight peptides than the corresponding free amino acids, with the competition between amino acids being abolished. These spatio-temporal events directly influence amino acid concentration in blood flow, which in turn affects many physiological and metabolic phenomena as well as influencing the overall rate of protein synthesis (Savoie et al. 2005).

As to the peptide and protein molecules that are larger than 6 amino acids, so they pass through the intestinal wall differently. There are several routes, which allow the more or less non-specific passage of molecules: specialized microfold cells of Peyer's patches, isolated lymphoid follicles, through the epithelial cells (transcellular pathway) and between these cells (paracellular pathway) (Perrier & Corthésy 2011). The mucosa-associated lymphoid tissue, comprising Peyer's patches and isolated lymphoid follicles, is covered by the follicle-associated epithelium containing microfold cells. These cells have the capacity to engulf particulate antigens, and transport them to underlying dendritic cells thus contribute

towards possible immune response. Paracellular transport across the epithelial barrier is highly regulated and is able to leave access to small molecules (44 angstroms), but also allows the passage of larger molecules including small peptides and bacterial lipopolysaccharides (500-1500 Da). A particular subset of dendritic cells (CX3CR11) is known to extend their dendrites in the lumen to collect bacteria under steady-state conditions, a process further accentuated upon inflammation. It is not yet clear whether this process can also take place for food antigens and whether this could contribute to the selective passage of antigens across the intestinal barrier and further presentation to the mucosal immune system (Perrier & Corthésy 2011).

Normally, large molecules are restricted from access to the intestinal wall by the mucus layer, which blocks passage of molecules of over 17 kDa (Jin et al. 2006). The intestinal cell layer permeability towards peptides and large molecules, on the other hand, is tightly bound to the external conditions. Thus, during fasting the efficiency of small peptide absorption increases (Silk et al. 1985). Then, a specific consequence of allergic reactions is the increased permeability towards large molecules via paracellular pathway, which can persist for a significant time after acute immune response is over (Perrier & Corthésy 2011). *In vitro* studies on confluent cultures of the enterocyte-like cell line Caco-2 have shown, that a model protein beta-lactoglobulin is taken by endocytosis, and whilst the majority of the protein was degraded intracellularly, around a third was transported across the cells (Wickham et al. 2009).

2.4 Comparison of relative roles of protein digestion stages

The functional importance of different stages of protein digestion process is unequal, albeit the exact significance of each of them varies depending on whether food toxicity/allergenicity or nutritive value is considered. As one could expect, the nutritive value of a protein is the most stable characteristic in context of the involvement of different digestive tract compartments. Thus, stomach and mucosal peptidases are dispensable for this function, and individual amino acid transport is also not very important if peptide transport is alive. The core functionality appears to be the one of pancreatic endopeptidases, which should split food protein into peptides of 3-2 amino acids; this activity is logically protected by the existence of multiple enzyme isoforms.

In addition to this, it should be noted, that peptide transport role is more impotant than the amino acid one. In evaluations involving both highly digestible proteins such as egg and less digestible protein sources such as corn as well as free amino acid mixtures, the food source of protein routinely gave better nitrogen balance test results than did the counterpart amino acid diets. Explanations for these results included the following: (a) that the immediate availability of purified amino acids might lead to overall overtaxing of absorption mechanism systems or competition among particular amino acids might lead to selected competitive inhibition of absorption of particular amino acids and hence to reduced efficiency of their utilization; (b) that rapid absorption of amino acids over a short period of time rather than over a longer period of time might overtax the body's ability to efficiently use amino acids for protein synthesis purposes, thus leading to increased deamination of amino acids and utilization for energy purposes; (c) that experimental diets containing purified amino acids were not truly matched to those containing food proteins in contents of all trace nutrients and results reflected these deficits (Kies 1981).

The situation changes significantly, as one tries to evaluate food protein toxicity and allergenicity. In this case stomach phase value greatly increases, as the low pH in this compartment often provides denaturation and thus inactivation and detoxification of ingested proteins. The oligopeptidases of the intestinal mucosa are not very important for the toxicity prevention, albeit they play a role as protectors against exogenous signals, as many proteins could possess hormonal properties (Widmaier et al. 2011). The pancreatic enzymes are still important for the defensive function, as they split large proteins into small chunks, thus destroying potential immunological epitopes.

3. The evaluation of protein and peptide digestibility

There are several approaches to the evaluation of protein digestibility. The most common of these include single-enzyme systems, based on pepsin and/or trypsin; whole-organ systems; cannula incision; and immobilized multiple digestive enzymes system. The latter model provides the most relevant data, while pepsin hydrolysis is the simplest. Several more rare models will be also described (for example, the use of cell fractions of small intestine, culture of Caco-2 cells, yeast surface enzymes, tissue fragments).

3.1 Isolated enzymes

One of the simplest models of protein digestion is the use of the purified major enzymes of stomach (pepsin) and pancreas (trypsin and chymotrypsin) (Horii et al. 2009; Huang et al. 2010; Kamata et al. 1982; Nielsen et al. 1988; Stanic et al. 2010; Yagami et al. 2000), implemented either separately of subsequently. The products of the digestion are usually analyzed using SDS-PAGE with the separation range from 3 to 20-30 kDa (Stanic et al. 2010; Yu et al. 2011) or reverse-phase HPLC (Bublin et al. 2008). In rare cases, mass spectrometry is also employed to characterize the digestion products (Dupont et al. 2010).

The procedure of pepsin and trypsin/chymotrypsin digestion is defined in the US and EU Pharmacopoeias (Council of Europe 2004; United States Pharmacopeial Convention 2006), and prescribes the exact composition of the incubation medium, enzyme to protein ratio and incubation time.

Several optimizations of enzyme digestion assay exist. First of all, the inclusion of non-protein components of gastric and intestinal juice, such as phosphatidyl choline and bile, and some potential food components (pectin) could significantly change the analysis result (Bublin et al. 2008; Dupont et al. 2010; Mandalari et al. 2009; Polovic et al. 2010). Not all proteins respond to such changes, but sometimes partially unfolded form of the protein is able to penetrate into the phosphatidyl choline vesicles. These interactions are probably responsible for slowing of gastric digestion by reducing the accessibility of the protein to pepsin (Moreno et al. 2005). Another option is the use not only of the major luminal proteases, but also of other enzymes: aminopeptidase M, leucine aminopeptidase, carboxypeptidase A and carboxypeptidase Y (Dizdaroglu et al. 1984; Tonglet et al. 2001), or modeling of the subsequent interaction of food protein with saliva, gastric juice and duodenal juice (Versantvoort et al. 2005), and heating during food processing (Nielsen et al. 1988), a group of approaches which should be definitively considered if some protein seems to be stable. Horii *et al.* implemented another interesting modification of enzyme digestion procedure. They introduced test protein into the incubation medium not as a solution of individual molecules, but as a component, expressed

on the surface of a whole yeast cell. The aim of this study was to check the stability of expressed oral vaccine, and it has shown that indeed, a protein displayed on the yeast cell surface had an increased resistance to pepsin, possibly due to pepsin underwent steric hindrance and only digested the outermost side of the yeast cell wall protein (Horii et al. 2009).

An unquestionable advantage of individual enzyme digestion method is its simplicity and speed, as the whole analysis takes only about 2 to 3 hours to complete (including the products separation) and does not require any specific instrumentation or animal procedures. Wickham *et al.*, however, notices, that such models «are particularly useful where there is limited digestion, e.g. stomach, but are less applicable for total digestion studies. These types of models are predominately used for digestion studies on simple foods and isolated or purified nutrients, and are therefore ideal for assessments of the digestibility of isolated allergenic proteins» (Wickham et al. 2009).

3.2 Isolated digestive tract compartments

An intermediate between *in vivo* and *in vitro* protein digestibility assays is the utilization of isolated digestive tract compartments (Polovic et al. 2010; Wickham et al. 2009). The experimental procedure requires an animal to be sacrificed immediately before the measurement. After that, the required compartment is surgically removed and either placed into a vial with a simulated fluid of this compartment, or sealed both sides with the salt mixture with substrate within. A variation of this technique implies the inversion of a digestive tract compartment inside out before sealing, so the resultant bag is put into a beaker with the substrate-containing solution, and the digestion products diffuse to the internal volume of such bag. Some researchers add glucose to the incubation medium to provide the tissue with energy.

The strong point of this approach is that the digestion and absorption systems of the intestine remain intact, and so the biological significance of the obtained results is very high, and the question on whether the digestion products really pass through the intestinal wall is immediately answered. The isolation of reaction products is also rather simple, as they are automatically separated from the source protein and mucosal proteins (if a sealed compartment bag is employed). The downsides of this method are, of course, high animal requirements and low suitability for high-throughput screening.

3.3 *In vivo* methods

In vivo methods, albeit the most complex ones, are at the same time the most informative ones. The two possible strategies within this group are based on the evaluation of either the remnants of the substrate within or at the end of digestive tract, or of the assimilation of the digestion products by the organism.

The methods based on protein nitrogen utilization by an organism are subject of several great reviews, and we refer a curious reader to them for more details (Bender 1958; Darragh & Hodgkinson 2000; von der Decken 1983). Below, a short description of method families will be given.

The first group of methods (protein efficiency ratio, net protein retention, rat-repletion method) use direct determination of the weight of animals, fed with a test protein. The

variations of this method lie in the employment of non-protein diet fed rats as a control group or starvation of animals before the introduction of a test meal (Bender 1958; Henry 1965). A variation of these methods suggests the determination not of the animal weight, but rather of amount of a specific tissue (liver, muscle, *etc.*), laid down while feeding on a test protein (Bender 1958; Mokady et al. 1969). These include protein retention efficiency and liver protein utilization. It should be noted, that the original growth determination methods are very animal-sparing, while the more advanced assays usually implicate animal death in order for some parameter to be determined.

Another group of methods, developed with the aim to overcome the not-so-obvious relationship between protein in diet and growth rate, introduces direct determination of nitrogen either in food before and after passage through digestive tract, or in animal carcass; a variant of latter analysis implicates the division of animal group in several parts with subsequent determination of carcass nitrogen at different time points (Bender 1958). The methods in this group are Thomas-Mitchell method, N-balance index method, carcass-nitrogen method and growth and nitrogen balance method. In some variations of nitrogen retention methods protein isotope labeling is required.

The further development of nitrogen analysis concept led researchers to the development of some biochemical methods for protein nutritive value determination. These methods are based on several parameters, which strongly correlate with tissue growth: polyribosomal profiles or ribosome activity and content; the activity of enzymes involved in urea metabolism or transamination reactions; 3-methylhistidine (an excreted-only myosin component) production and creatinine excretion (Bender 1958; von der Decken 1983). Ribosome activity is quite informative, as it is sensitive enough to detect small differences in protein quality. The latter two parameters, on the other hand, are measured in urine and thus are suitable for humans.

The current recommendation, when calculating a protein digestibility, is to determine the digestibility of a dietary protein across the entire digestive tract, using the rat as a model animal for humans. This fecal digestibility value is subsequently corrected for endogenous contributions of protein using a metabolic nitrogen value determined by feeding rats a protein-free diet, a task for which the protein digestibility-corrected amino acid score (PDCAAS) was introduced. To calculate a PDCAAS, the availability of the amino acids in a dietary protein is assessed based on the digestibility of total nitrogen (N) in that dietary protein. Digestibility is defined as the difference between the amount of N ingested and excreted, expressed as a proportion of N ingested. Although accepted as the recommended procedure, the use of fecal digestibility coefficients to evaluate amino acid availability is thought to be inherently inaccurate due to the metabolism of both dietary and endogenous proteins by the hindgut microbial population (Darragh & Hodgkinson 2000; Kies 1981).

In fact, the aforementioned techniques are not precisely suitable for protein digestibility evaluation. The problem is that they measure protein assimilation and do not provide any data on how exactly a particular protein was or was not digested and transported. This hindrance is circumvented in the second group of *in vivo* approaches, which include swallowed probe utilization, anastomosis and cannula incision (Darragh & Hodgkinson 2000; Faber et al. 2010).

Anastomosis involves transecting the ileum anterior to the ileocecal junction and attaching this to the descending colon. This allows a quantitative collection of ileal digesta, but many

studies have shown that anastomosed animals have an altered physiology compared with intact animals. It should be noted that human ileostomates may also have an altered physiology compared with intact humans, which could also call into question the validity of using human ileostomates for the collecttion of ileal digesta (Darragh & Hodgkinson 2000).

The insertion of a re-entrant cannula involves transecting the terminal ileum and sealing the two ends. A cannula is inserted into each end of the sealed ileum and the two cannulae are joined. This allows a quantitative collection of digesta. The surgery to insert a re-entrant cannula is complex, however, and involves total transection of the ileum. Blockage of the cannula is a common complication. Simple T-piece cannulation and postvalve T-cecum cannulation have the distinct advantage of maintaining the ileocecal valve intact and avoiding ileal transection; in this case there is also no surgical interference with the small intestine. In addition to that, most of the digesta should pass through such cannula during sampling, as the ileocecal value protrudes directly into it (Darragh & Hodgkinson 2000).

The *in vivo* methods of protein bioaccessibility determination, such as retained nitrogen analysis, cannula incision and swallowed probe sampling, undoubtedly provide the most relevant data. However, their major drawback is experimental complexity and practical impossibility of any reasonable screening.

3.4 Digestive tract simulators

Most of the aforementioned models, excluding the animal ones, lack two important characteristics of digestive tract — the mechanical forces, exerted by stomach and intestinal walls, and the fact that food arrives into stomach and upper intestine not as a solution or an emulsion, but rather as a bolus with different accessibility of its internal and external contents for the enzymatic machinery. The digestive tract simulators try to overcome this situation. Usually they represent complex devices, consisting of several mixing chambers with programmatically controlled pH, mixing speed, temperature and valves between them, which provide a definite transition time from one compartment to another. The compartments that imitate intestine contain immobilized enzymes, either trypsin and chymotrypsin, or more complex mixtures including proteases of intestinal wall. The system is filled with simulated gastric and intestinal fluid plus phospholipids and/or bile, and the test substances are delivered into the system either in form of solution or within agarose beads (a way to mimic food bolus). The result of protein digestion in such system could be analyzed at any stage; the commonly employed analytical methods are SDS-PAGE (mass range 2-15 or 14-60 kDa) and reverse phase HPLC.

The most representative example of such device is the Dynamic Gastric Model, described by Vardakou et al. *(*Vardakou et al. 2011*)*. It is composed of three parts, the main body (fundus), the antrum and the valve assembly. In the main body of the model the inhomogeneous mixing of the stomach is reproduced by gentle contractions induced by computer-controlled changes in the applied pressure of water in the thermostated water bath surrounding the main body. Gastric acid and enzymes are added from a dispenser that is floating on the top of the main body contents. The dispenser is designed in such a way as to deliver the enzyme and acid evenly from the sides of the main body, replicating the human gastric secretions originating from the walls of the stomach. The rate of addition of both enzymatic and acid secretions is also computer-controlled. The food material is

allowed to move from the main body into the antrum, and *vice versa*. The artificial antrum simulates the strong shear forces of the human antrum to reproduce the breakdown of the food particles and the preferential sieving observed *in vivo*. The mechanical processing of the food within the antrum is achieved by the sliding of a piston within a barrel, which forces the material through an elastic annulus where selective sieving takes place. Once ready, the processed bolus is ejected through the valve assembly and can be collected for further analysis.

Another widely used system is the Immobilized Digestive Enzyme Assay (IDEA), developed by Schasteen et al. (Schasteen et al. 2002). This system is somewhat more simple, and concentrates not on the grinding forces, but rather on the mixing of the «ingested» components and the food transition times through various digestive tract compartments. The overall digestion procedure consists of a stepwise acid solubilization, pepsin digestion, neutralization, trypsin, chymotrypsin and intestinal peptidase digestion followed by analysis of hydrolysis products. As follows from the model name, it relies on the glass beads-immobilized enzymes, and this allows for substantial costs reduction. The downside of this particular model is very long analysis time — up to 2.5 days.

The digestive tract simulators possess several advantages. They are more efficient at predicting of the fate of tablets within the gastrointestinal tract and more accurate at the simulation of the interaction of investigated protein or peptide with other meal and chyme components. The use of immobilized enzymes allows the reduction of experiment costs. However, the pay-off of these systems lies within their design: the complete analysis procedure usually takes a day or even two, and the complexity of the system dramatically reduces the throughput, as usually only one protein could be analyzed by one device at once. Finally, such machines are usually too complex to build by a research team, and thus require a substantial capital investment at the initial stage of a research project.

3.5 Other approaches

Beyond aforementioned widely used approaches to study of protein digestibility, several less common or hybrid ones also exist.

First of all, when it comes to the evaluation of protein nutritive value, the simplest way to determine it is to calculate relative quantity of essential amino acids in the test protein relative to some well-known food protein. Hansen et al. (Hansen 1975) describes two such indices: Essential Amino Acid Index (EAAI) and Arnoulds index. The reference protein in both cases is egg protein; the latter index differs by including of the sum of the non-essential amino acids (NEAA) as part of equation. An unquestionable advantage of the amino acid scores is very low requirement of experimental work for production of preliminary estimates about a protein. However, this method does not take into account any properties of a protein, thus making impossible to draw any conclusions on its real bioaccessibility.

Then, von der Decken et al. point out to the fact, that the protozoan *Tetrahymena pyriformis W* has a proteolytic enzyme to digest proteins and similar to human needs for essential amino acids, and thus could be a model for protein digestion (von der Decken 1983). Time consumption of such model is, however, rather high (48 to 66 hours), and the details of animal digestion process are missed as well.

Our group has recently suggested an assay, that tries to replicate the conditions of parietal digestion in small intestine, at the same time maintaining capability for high-throughput sample analysis (Akimov et al. 2010). The active component of this system is a fragment of rat stomach or intestinal wall, immersed in simulated gastric or intestinal fluid. The products are analyzed by HPLC. The utilization of tissue fragments allows for reduction of animal consumption, while still maintaining the whole set of intestinal peptidases and providing some phosphatidyl choline in the medium. The assay design is suitable for the processing of 40 samples per day.

To simultaneously determine protein digestibility in small intestine and transport through epithelial cells, a hybrid model is often employed (Dhuique-Mayer et al. 2007; Jin et al. 2006; Versantvoort et al. 2005). The intestinal epithelium is represented by the Caco-2 cell culture, which is grown to the monolayer state on a membrane that separates two chambers. A test protein solution with digestive enzyme or after *in vitro* digestion is applied in one chamber and, if transport is possible, the products are collected from the second chamber. Two optimizations of this experimental design have been proposed: first, a mucin layer could be applied on the surface of the cell culture, thus mimicking the real mucus layer, which exists in intestine. Mucin protects cells from damage by luminal digestive enzymes and limits the passage of large (over 1500 Da) molecules to the cell surface (Jin et al. 2006). Second, Caco-2 cells could be co-cultured with HT29-MTX cells, which imitate mucus-producing cells of small intestine; this approach could be considered a more natural variant of previous modification (Yao et al. 2010). The core complication of such methods is the requirement for the facilities for operations with animal cell cultures. On the other hand, the model could be used for medium-sized screening, has a decent degree of intestinal wall imitation and thus the obtained results are quite relevant.

The final and most promising approach is the molecular modeling. In a work by Foltz et al. (Foltz et al. 2009) a special database was constructed to study the relationship between peptide structure and activity, permeability, and digestive stability. For this purpose, a total of 228 dipeptides were synthesized and their intestinal stability was evaluated by *in vitro* digestion. Then, a quantitative structure-activity relationship (QSAR) modeling was performed using partial least squares regression based on 400 molecular descriptors. The correlation coefficient for the best fit model was 0.76, and proteolytic stability for 12 new peptides was successfully predicted. As it is seen from the description above, molecular modeling has one major drawback: it requires a huge amount of preliminary data to build an efficient model. After that, it offers great speed and low experimental costs, but the aforementioned initial stage makes this approach suitable only for large screening experiments.

4. Current view of protein stability evaluation in different fields

The common approaches to the determination of the protein and peptide digestibility in different areas of research varies significantly:

- Allergenicity studies are usually the simplest ones and rely on direct hydrolysis of test substrate by pepsin and a combination of trypsin with chymotrypsin in simulated gastric and intestinal fluids.
- Biological value is most commonly determined using animal assays.

- The studies on the properties of digestive enzymes rely on the purified enzymes or on fractionated homogenates of the parts of gastrointestinal tract.
- Pharmacological studies tend to use complicated devices for the evaluation of the ability of a particular pharmacological form to degrade in specific compartment of digestive tract.

In fact, the stability towards protease degradation is not considered to be a basic method for the evaluation of potential protein allergenicity, albeit the resistant proteins are usually allergenic (Fu 2002). To better understand the data on the digestibility of any potential immunogenic peptide, a basic picture of the underlying mechanisms is necessary (for more details reader is referred to an excellent review by Huby et al. (Huby et al. 2000)).

Allergic sensitization to proteins involves the induction of IgE type antibodies production of sufficient magnitude by differentiated B-lymphocytes to facilitate the elicitation of an inflammatory reaction following subsequent exposure to the same (or a cross-reactive) allergen. For a B cell to differentiate into a plasma cell and produce antibodies, the B-cell receptor expressed on its surface must bind to specific B epitopes on the surface of the protein antigen. The antibodies subsequently produced by the plasma cell, into which the B cell differentiates, have the same specificity as the B-cell receptor, and are therefore able to bind specifically to the same B epitopes on the surface of the protein antigen. Efficient secretion of antibodies normally requires that the B cells receive help from T-helper cells that specifically recognize separate epitopes on the same protein antigen. Such recognition is mediated by the T-cell antigen receptor, which delivers stimulatory intracellular signals to the T cell. Allergenic proteins *per se* cannot be recognized by T cells; the proteins must first be processed and then presented by specialized antigen-presenting cells (APCs) (Gorovits 2010; Huby et al. 2000).

After the first contact with an antigen the T-helper-stimulated B-cells differentiate and become ready to produce antibodies. Thus, if such antigen is encountered for the second time, there will be some antibodies against it present in the blood flow, and the production of their large quantities will start immediately. The IgE antibodies, however, serve mainly as information transducers during allergic response. After antigen recognition they bind to the Fc receptors for IgE (FceR) on the surface of basophils and mast cells; for these cells to become activated and secrete histamine and other inflammatory mediators their receptors need to dimerize. Thus, the peptide is to be large enough to bind at least two antibodies, which will bring two FceR together. The minimal length of peptide chain, which could be recognized by IgE antibody (epitope), is 15 amino acids, and thus the minimum size of an immunogenic peptide should be around 30 amino acids or 5-6 kDa (Van Beresteijn et al. 1994). At the same time, the sensitization stage, when an antigen is recognized by T-cells and undifferentiated B-cells, requires only one epitope and thus a peptide size of around 3 kDa (Moreno 2007; Van Beresteijn et al. 1994).

The discussion above clearly indicates the valid conditions of digest products analysis: HPLC and MALDI are always informative enough, but SDS-PAGE is appropriate only if it includes molecular masses of 2-3 kDa, which unfortunately is not always the case (Huang et al. 2010; Yagami et al. 2000; Yu et al. 2011).

Even after all the requirements for the stability assay of a potential allergen are met, the results should be interpreted with caution, as some stable proteins are not allergens at all,

and some other, which are completely degraded in the experimental conditions, still induce immune response *in vivo* (Faeste et al. 2007; Fu 2002; Jensen-Jarolim & Untersmayr 2006).

One of the complicated moments during the immune response is that depending on the type of T-cell, the type of antibodies produced by the B-cell could be changed: Th2 cells produce a cocktail of cytokines that, among other actions, encourage plasma cells to switch to synthesis of IgE, while Th1 cells typically produce interleukin 2, interferon gamma, and tumor necrosis factor (TNF)-alpha/beta, that together downregulate IgE synthesis. Only one type of stimulation could be active for a given antigen, as Th1 cytokines suppress Th2 cell development, and *vice versa*. Structural investigations of several allergens have identified T epitopes implicated in the selective development of Th2-type lymphocytes. Some allergens, such as ovalbumin, have only a few epitopes that preferentially induce a Th2 response, whereas others, such as beta-lactoglobulin, have many. The potential to induce a polarized (Th1 or Th2) response is not exclusively an intrinsic property of particular epitopes; the quality of the T-cell response to any given epitope depends upon many factors, including the dose of protein or peptide given, the affinity of the peptide for MHC class II receptor, and the longevity of the class II/peptide complex. Together, these parameters act to influence the dynamic epitope density on the surface of APCs. It is generally agreed that parameters that serve to increase ligand density, including greater affinity to MHC class II, higher levels of ligand loading, or longer-lived complexes favor Th1-type responses (Huby et al. 2000).

In addition to the possible anti-allergic action of Th1 epitopes, a significant variety into experimental results is introduced due to the experimental conditions differences. Several investigators term protein as «stable» after incubation times ranging from 8 to 60 min (Bannon et al. 2003; Fu 2002; Herman et al. 2006). As far as the unstable proteins usually survive less than 15 sec in the similar assay conditions, such conclusion seems to be quite correct. However, the 8-min survival could potentially be an indication of the protein instability, as the simplified conditions of simulated gastric and intestinal fluid do not take into account intestinal mucosa peptidases or minor luminal endopeptidases. Then, albeit standardized in the last period, the enzyme to substrate ratios vary between different research groups (Herman et al. 2006), and thus obtained stability or instability may not adequately reflect the reality.

Finally, the conditions inside the digestive tract during the potential allergen ingestion are of crucial importance. For example, during inflammation the permeability of digestive tract is increased (Perrier & Corthésy 2011); then, any elevation of the pH should result in hindrance of peptic degradation. Numerous situations with elevated stomach pH are known, e.g. in early childhood, in elderly, or in chronic atrophic gastritis. Moreover, there is a number of pathologies, like gastritis or ulcer, where acid neutralization or inhibition is an important therapeutic goal. Acid neutralization is state of the art during surgical care, corticosteroid or analgesic treatment. Moreover, anti-acids, H2-receptor blockers and proton pump inhibitors are increasingly consumed without prescriptions due to liberalization of the market by over-the-counter sale (Jensen-Jarolim & Untersmayr 2006).

As a logical consequence of the aforementioned complications, immunogenicity assessment is typically done using a multistage strategy, generally including an digestive stability and antibody affinity screening assay, a specificity confirmation step, and in some cases a

characterization step. Samples are first evaluated in a screening assay, for which an assay threshold has been set based on the variability of samples from a drug naïve target patient population. Screen positive samples are further evaluated in a confirmatory assay to verify whether the signal observed in the screening assay is a result of a specific response to the protein therapeutic treatment. Confirmed positive samples are then put into downstream methods for sequential characterization based on the comprehensive consideration of immunogenicity risk assessment and mechanism of action for the protein therapeutic (Gorovits 2010; Peng et al. 2011).

The studies of protein nutritive value usually rely on the *in vivo* experiments or on the data on protein amino acid composition. The point is that the most important aim of such studies is to tell, whether a given protein (perhaps after some processing) is suitable for supplying body needs for energy and essential amino acids. The question on how does an organism manage to consume that protein is often ignored, albeit it is tempting to speculate that the exact metabolic route from the whole protein to the amino acids and energy inside body cells could be important as well. One should note that as it was shown in section 2, there are several conceptually different steps in protein digestion (denaturation and splicing in large chunks in stomach, splicing in tetra- to dipeptides and individual amino acids in small intestine and various processing by the large intestine microbiota), whose efficiency could be independently affected by the protein structure and modifications, and thus during some pathological conditions a protein of a high nutritive value could suddenly lose it.

The *in vivo* evaluation of nutritive value consists of animal feeding with test protein with subsequent analysis of either the amount of absorbed or unabsorbed protein fractions, or the degree to which such meal is able to sustain animal growth. The experiments are usually quite long, expensive and involve complex manipulations with animals. The direct determination of protein amino acid composition is much simpler. The only complication of such approach is that the degree to which the constituent amino acids of a food protein are actually available to the body is determined by such factors as protein configuration, amino acid bonding, other constituents of the diet, and the physiological condition of the gastrointestinal tract of the individual person or animal involved. However, the correlation between prediction and performance is quite good: prediction for poor performance of proteins devoid or nearly devoid of an essential amino acid as well as of good performance for proteins containing all essential amino acids according to idealized patterns is excellent. However, fine-line predictions of intermediate quality are less accurate, and surprises are not uncommon (Kies 1981). Thus the *in vivo* models are still a preferred way for nutritive value analysis.

Enzymatic studies are usually concerned not with the stability of a some protein, but rather with the distribution, specificity and kinetics of a particular enzyme, and thus they usually implement some procedures of purification from animal tissues (Krzysik & Adibi 1977; Smith & Bergmann 1944; Sterchi & Woodley 1980a) or of expression of a recombinant enzyme in some prokaryotic or eukaryotic cells (Hauri et al. 1985; Lentze 1995). The applicability of such procedures is usually quite legitimate, albeit the biological significance and theoretical value of the obtained results could vary. The enzyme-to-substrate ratios in such studies usually do not resemble the *in vivo* situation, and thus the real significance of a given enzyme for the digestive process could not always be predicted. The differences of purification procedures complicate the integration of data from various research groups into

Name	Reproduced conditions	Time to complete and special conditions	Reference
In vitro methods			
Simulated gastric fluid	Stomach lumen without mechanical forces after individual ingestion	Hours	(Thomas et al. 2004)
Simulated intestinal fluid	Intestinal lumen without shed mucosal cells and mechanical forces after individual ingestion	Hours	(Tonglet et al. 2001)
Amino acid score	Ability of protein to be a source of essential amino acids	Hours	(Hansen 1975)
Organ fragments	Mucosal and intracellular digestion	Hours; HPLC or MALDI analysis	(Akimov et al. 2010)
A device with immobilized enzymes	Luminal digestion with mechanical forces ant transitional times without mucosal enzymes	Hours (single compartment) to days (whole digestive tract); requires a special device	(Vardakou et al. 2011)
Ex vivo methods			
Caco-2 cell culture	Luminal and intracellular intestinal digestion, transport	Hours + cell culture preparation; requires animal cell culture facilities	(Dhuique-Mayer et al. 2007)
Isolated organs	Luminal, mucosal and intracellular digestion of a given organ, transport	Hours	(Curtis et al. 1978)
Bacterial culture	Microbial digestion in large intestine; ability to provide essential amino acids	Hours; requires microbiological facilities	(Bender 1958)
In vivo methods			
Nitrogen balance	Whole digestion process in a given health condition	Days-weeks; may require isotope labeling	(Mokady et al. 1969)
Cannula/probe	Digestive process up to the point of probing without data on digestion products	Days(cannula); hours(probe); applicable for human	(Darragh & Hodgkinson 2000; Faber et al. 2010)
In silico methods			
QSAR	Predicts degradation in any conditions depending on model	Minutes (computation), weeks to months (database and model development); requires large experimental body (data on 100-200 proteins)	(Foltz et al. 2009)

Table 2. A comparison of different method classes for protein digestibility evaluation

one picture (*cf.*, for example, the discussion in the work by Sterchi et al. (Sterchi & Woodley 1980a)).

The aim of a substantial part of pharmacological studies of peptide- and protein-based therapeutics is often the evaluation of the disintegration, dissolution and drug release profiles of oral drug formulations (Vardakou et al. 2011). Thus another aspect of food digestion becomes important – the mechanical grinding and mixing forces, exerted on a pharmaceutical formulation by various digestive tract compartments. To simulate such conditions devices of various complexity are employed (Schasteen et al. 2002; Vardakou et al. 2011). The applicability of such devices for the drug form disintegration testing is unquestionable, however, usually they reproduce only the luminal phase of food digestion. Thus the evaluation of the ability of released protein or peptide to get to the blood flow intact requires additional methods.

A short reference of available method types is provided in table 2. It should be noted, that some *in vitro* methods could be combined and expanded to provide more relevant data. For example, single-enzyme digestions by stomach and small intestine could be performed sequentially, the Caco-2 cell culture is often overlaid by a simulated gastric fluid and possibly mucin (to protect cells and imitate the similar layer present *in vivo*). The computational methods are the most adaptive and powerful ones, however, to date their usability is limited to large screening studies due to huge preparatory experimental work.

5. Conclusion

Significant progress has been made in the modeling of protein digestion and absorption in gastrointestinal tract since the first works on this topic in the beginning of the XX century. A multitude of methods of various complexity, biological significance, animal requirements and human applicability now exist to answer the question on digestibility of proteins and peptides. The degree of biological processes imitation ranges from single luminal enzymes in simulated gastric or intestinal fluid model to sophisticated devices, which take in account grinding, mixing and turbulence forces and transition times, as well as isolated animal organs.

There is, however, no single method, which will suit every possible research goal. The problem is, that as the biological significance increases, time and labor requirements also rise tremendously, and methods became progressively less suitable for high throughput screening, which is often required. In addition to this, there is no experimental model, which could imitate every last component of the digestion process. The enzyme-only models and organ fragments ignore mechanical forces, digestive devices lack intracellular hydrolases and transport steps, isolated organs usually represent only one compartment of the whole system, and they fail to exert mechanical forces as well. As there is no efficient methodology of sampling of the digested products after they are absorbed, but before they enter blood or lymph, whole animal models are also limited – they can only answer questions, whether a protein was absorbed and if it was suitable for the sustaining normal growth of an organism. A very promising approach has appeared with the development of the high-performance computation methods, that is, molecular modeling. Theoretically, mathematical modeling is able to join data on the digestion patterns of various proteins from *in vitro* experiments with the results of *in vivo* tests of the ability of an organism to

utilize these proteins. However, a vast array of preliminary data is required for optimization and validation of such models, and so they are relatively rare.

Thus, when considering various methods of protein digestibility assay, one should first of all define the limitations of the research at hand. If a high throughput screening is required? If there is a need for any data on digestion products? If absorption efficiency could vary and is essential? If a protein should serve as food or not? A possible decision tree for method optimization is presented on figure 1, albeit it should be considered more as a reference than a rule.

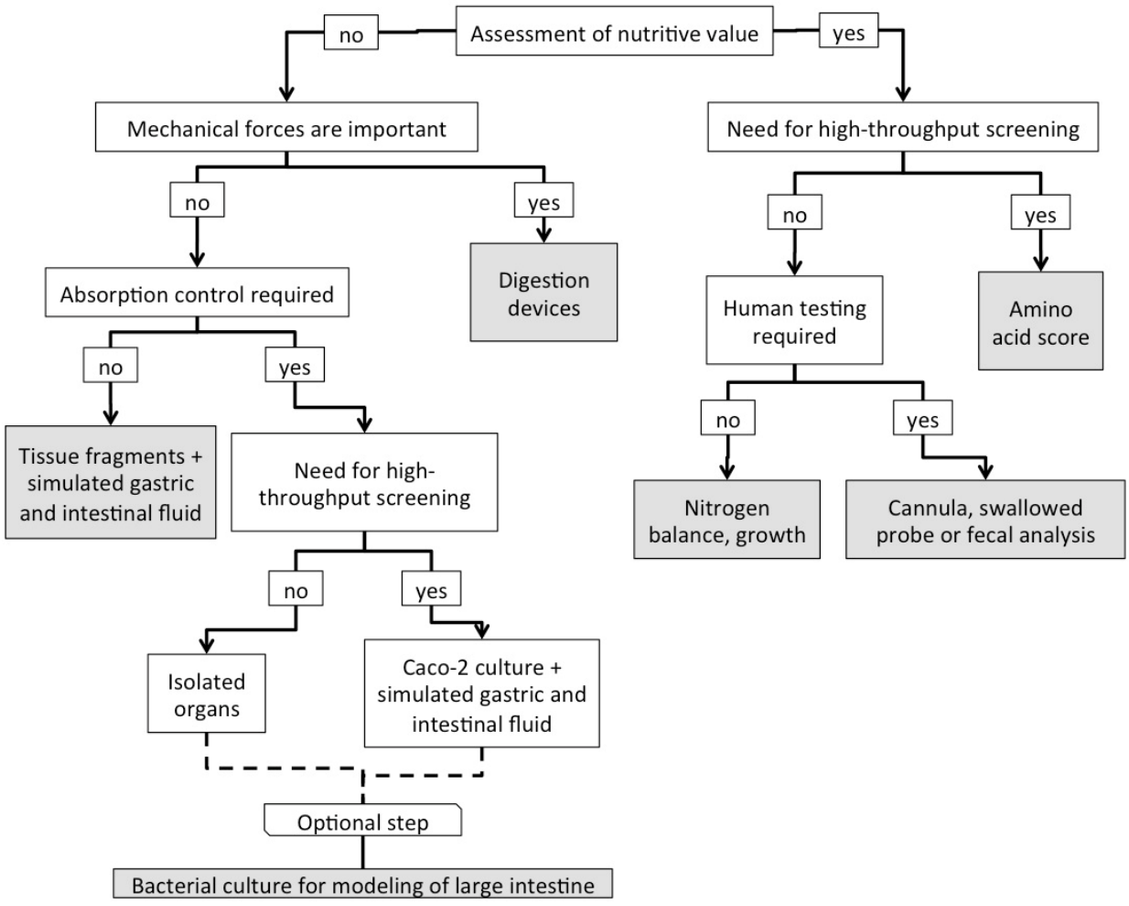

Fig. 1. A decision tree for protein or peptide digestibility assay choice

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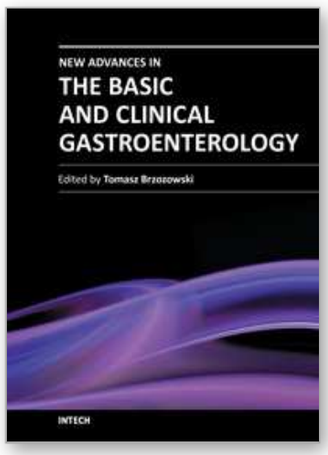

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The purpose of this book was to present the integrative, basic and clinical approaches based on recent developments in the field of gastroenterology. The most important advances in the pathophysiology and treatment of gastrointestinal disorders are discussed including; gastroesophageal reflux disease (GERD), peptic ulcer disease, irritable bowel disease (IBD), NSAIDs-induced gastroenteropathy and pancreatitis. Special focus was addressed to microbial aspects in the gut including recent achievements in the understanding of function of probiotic bacteria, their interaction with gastrointestinal epithelium and usefulness in the treatment of human disorders. We hope that this book will provide relevant new information useful to clinicians and basic scientists as well as to medical students, all looking for new advancements in the field of gastroenterology.

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