

In vitro determination of dietary protein and amino acid digestibility for humans

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Abstract

The development, refinement and validation of *in vitro* digestibility assays for dietary protein and amino acids for single stomached mammals are reviewed. The general principles of *in vitro* digestibility assays and their limitations are discussed. *In vitro* protein digestibility assays must be accurate, rapid, cheap, simple, robust, adaptable and relevant to the processes of digestion, absorption, and metabolism. Simple *in vitro* methods have the potential to give useful measures of *in vivo* amino acid and protein digestibility for humans. *In vitro* methods, including the complex multi-component models of digestion simulating the various physical and chemical processes, require independent validation with *in vivo* data from the target species or an acceptable animal model using the most appropriate *in vivo* measure of digestibility. For protein sources devoid of anti-nutritional factors or plant fibre, true ileal digestibility is the recommended *in vivo* baseline, while for plant proteins the recommended *in vivo* assay is real ileal digestibility. More published comparative studies are required to adequately validate *in vitro* digestibility assays.

Key words: Digestion: Digestibility: Protein: Amino acids: Humans

Humans require many nutrients including dietary indispensable amino acids, vitamins, minerals and fatty acids. The dietary indispensable amino acids required by humans are provided to the body mostly as intact dietary proteins which require digestion to release their component amino acids and small peptides. Proteins vary in their content of constituent amino acids and can be devoid of or low in one or more dietary indispensable amino acids, and when fed as the sole source of amino acids cannot sustain life. The biological utilisation of a protein is influenced by its composition, particularly with respect to the dietary indispensable amino acids, its digestion in the gastrointestinal tract, and the absorption and transport of amino acids and peptides from the gastrointestinal tract. Processing and storage conditions of a food affect the interactions between the components, with effects on digestibility that can have both beneficial and detrimental effects on protein nutritional quality.

Digestion and absorption processes in the live animal are complex, highly integrated and regulated, and are adaptable processes that have evolved to efficiently release nutrients for the body's growth, maintenance and reproduction. These dynamic processes are under both neural and hormonal control and respond to various stimuli. To simulate such a complex system in its entirety using static unresponsive *in vitro* methods is very difficult if not impossible⁽¹⁾ and

therefore how *in vitro* assays are applied should be tempered accordingly. Also, the effects of the gut microbiota are particularly difficult to simulate as is the diverse impact anti-nutritional factors and dietary fibre have on the digestive tract and its processes. Yet *in vivo* measures are expensive and time consuming. Therefore there is a need for a rapid reproducible *in vitro* digestibility bioassay that provides a reliable estimation of digestibility for a wide range of foods.

The development and practical details of *in vitro* procedures have been previously reviewed^(2–5). *In vitro* protein digestibility assays and their validation with *in vivo* measures have been reviewed by Moughan⁽⁶⁾. An overview of *in vitro* digestion models for food applications has recently been published by Hur *et al.*⁽⁷⁾. The present review summarises recent developments in *in vitro* digestibility bioassays and their validation with *in vivo* measures.

In vitro methods

In vitro digestibility methods began as simple one step incubations with pepsin or other proteases such as trypsin, papain, pronase or rennin⁽²⁾. These early single enzyme methods appeared to be satisfactory for comparing the effects of various treatments on a single foodstuff but gave lower digestible protein values than those obtained *in vivo* (faecal

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nitrogen digestibility). Other methods have simulated gastric and intestinal digestion using a 2-stage *in vitro* digestion involving digestion in a pepsin-HCl mixture followed by neutralisation, and then digestion in pancreatin^(8,9), trypsin⁽¹⁰⁾, or intestinal fluid from pigs⁽¹¹⁾. In general, there has been good agreement between these *in vitro* results and *in vivo* rat true faecal nitrogen digestibility.

Multi-enzyme processes that measure the pH drop after a 10 minute digestion have been developed^(12,13). Good correlations ($r > 0.8$) were found with *in vivo* digestibility (rat true faecal) particularly when the protein sources were analysed by plant or animal origin^(14,15), and this method was found to be highly reproducible across six laboratories⁽¹⁶⁾. This approach assumes that the rate of change in pH is correlated with protein digestibility and that there is a direct relationship between the observed pH drop and the extent of protein hydrolysis^(17,18). The components of some food materials, however, interfere with the pH drop due to their buffering capacity^(14,19,20).

A modification of this approach is the pH stat method where the pH is kept constant by automatic titration with 0.1 M NaOH and the total amount of alkali used at the end of the incubation is recorded. This modification improved the prediction of *in vivo* protein digestibility, was reproducible, and gave high correlations ($r > 0.90$) with *in vivo* (rat faecal) digestibility for highly digestible animal protein sources⁽¹⁵⁾.

The immobilised digestive enzyme assay (IDEA) system⁽²¹⁾ uses a bioreactor containing enzymes immobilised on glass beads, eliminating contamination of the digest with digestive enzymes and preventing autolysis. However, this assay takes two and a half days to complete so is not as rapid as other *in vitro* techniques. Predicted digestibility values from the IDEA assay have been found to correlate ($r = 0.8, 0.83$) with rat faecal protein digestibility for a range of foods^(22,23).

Gauthier *et al.*⁽²⁴⁾ developed an *in vitro* digestion under constant dialysis (molecular weight cut off 1000 Daltons) with specialised apparatus (dialysis cell method) to address the concern that enzyme activity is suppressed by the products of digestion. Continuous dialysis, however, needs extra and complex equipment, and some researchers do not regard it as necessary^(3,9). The proteins undigested following a pepsin-pancreatin digestion were determined using polyacrylamide gel electrophoresis (PAGE), allowing the estimation of the molecular weight of the polypeptides and proteins remaining after hydrolysis⁽²⁵⁾. This procedure was time consuming, taking 5 days to complete, and is likely too labour intensive to be used in a rapid routine assay.

Near-infrared spectroscopy (NIRS) is a very rapid technique with low maintenance costs that shows great promise in evaluating nutritive value for human foods. It has been used routinely for determining the chemical composition, organic matter digestibility, energy digestibility of grains^(28,29) and oil seeds⁽²⁶⁾ for monogastric animals. There is a requirement, however, for a large number of reference samples to calibrate the instrument and these reference data must come from *in vivo* digestibility assays. There have been only a few published studies comparing NIRS and *in vivo* nitrogen or protein digestibility values. Prediction of wheat ileal crude protein

digestibility for broilers⁽²⁷⁾ was found to be highly variable ($r^2 = 0.23-0.76$). Faecal digestible protein of commercial dry extruded foods for dogs showed good predictions using the pH drop ($r^2 = 0.78$) method, and *in vitro* two-step digestion ($r^2 = 0.81$), but not for NIRS ($r^2 = 0.53$)⁽²⁸⁾. Pig ileal nitrogen digestibility of barley ($r^2 = 0.97, 0.72$)^(29,30) but not wheat ($r^2 = 0.22$)⁽³¹⁾ has been accurately predicted with NIRS.

A complex biochemical model of human adult and infant gastro-duodenal digestion has been developed^(32,33) to investigate the allergenic potential of protein digestion products. Electrophoresis, immunochemical and mass spectrophotometry techniques were used to characterise the digestion products. This study found that heat treatment of milk increased the resistance of casein to the simulated digestion processes. An inter-laboratory study⁽³⁴⁾, comparing the digestion of β -casein and β -lactoglobulin using a simulated human gastro-duodenal *in vitro* assay, found it to be reproducible.

A computer-controlled, dynamic, multi-compartmental model (TIM) that seemingly closely simulates the conditions of the *in vivo* gastrointestinal tract of humans and monogastric animals has been developed at the TNO Nutrition & Food Research Institute, The Netherlands⁽³⁵⁾. It comprises compartments with flexible walls heated by water jackets with computer controlled pH adjustment, and rotary and peristaltic pumps to provide mechanical movement of the chyme. It simulates gastric pH change, peristaltic movement, gastric emptying rates, intestinal transit times, gastric, biliary and pancreatic secretion and their activities, small intestinal absorption (TIM-1), and the absorption from the large bowel (TIM-2) of volatile fatty acids and water^(35,36). This model has been applied in one published protein digestion study to date⁽³⁷⁾ in which the model was modified to simulate the infant gut and to measure the degree of protein hydrolysis of whey protein hydrolysate infant formulas. The hydrolysis of the proteins was found to be only one factor in determining the digestibility of these food products. TIM has been used to investigate nutraceutical delivery vehicles⁽³⁸⁾, gut transit time and iron absorption⁽³⁹⁾, and the availability of heterocyclic aromatic amines⁽⁴⁰⁾, and has been used in pharmaceutical studies⁽⁴¹⁾. One of the strengths of these models is their flexibility and ability to model different gastrointestinal conditions such as those found in infants^(32,33,37), or other animal species^(42,43).

A similar system to TIM is the Institute of Food Research, United Kingdom, dynamic gastric model (DGM) comprising two parts that mimic the fundus and antrum of the stomach, and this is integrated with a simulated intestine that mixes the digesta and adds bicarbonate, bile and digestive enzymes⁽⁴⁴⁾ and has also been used in pharmaceutical studies. Model stomach⁽⁴⁵⁻⁴⁷⁾ and small intestine⁽⁴⁸⁾ systems have also been developed to better understand the mechanical forces and mass transfer kinetics of food digestion and absorption. To date these systems have not been used for the routine evaluation of protein or amino acid digestibility of foods.

These complex computer controlled systems are expensive to set up and maintain and therefore may not be a useful tool for the routine evaluation of food. Although being far more sophisticated than simple digestion assays, they do not provide an entirely accurate reproduction of the physiological

system whereby the digesta interact with the gut cells leading to active transport of nutrients; nor do they simulate the neural and hormonal feedback mechanisms affecting digestion and absorption⁽⁴⁹⁾. Their validity for predicting the *in vivo* digestion of food proteins has not yet been fully tested. The choice of a standard method will be a balance between accuracy and ease of use, and the endpoint may simply be to rank foods in order of nutrient digestibility. Multiple regression equations combining *in vitro* digestibility coefficients and chemical constituent measures may have application where greater accuracy is required.

Limitations of the *in vitro* approach

In general, there are some key requirements for the development of *in vitro* digestibility assays: matching *in vivo* enzymes in presence, sequence, enzyme:substrate ratios; standardising enzyme activities and specificities; controlling co-enzymes and co-factors, pH and temperature; separating digested from undigested material while considering the inhibition of end products on digestion; and allowing for the effects of sample size, particle size and particle size distribution⁽⁶⁾. Digestion and absorption involve complex physiological processes that are virtually impossible to reproduce *in vitro*^(2,50). The effects of anti-nutritional factors, dietary dry-matter and fibre, endogenous protein secretions, activity of gut enzymes, and gut bacteria are not able to be mimicked *in vitro*⁽⁶⁾. The sensitivity of an assay is a function of both the time of the reaction and the enzyme-substrate ratio^(51,52). *In vitro* assays may need to include lipases, carbohydrases, and elastases to better mimic *in vivo* digestion and the release of proteins from the food matrix. There is an assumption that all soluble material is digestible whereas small peptides may not be absorbed *in vivo* particularly in heat-treated proteins.

An *in vitro* method may be precise and reliable in the laboratory but the resulting data must be correlated with *in vivo* data to provide a physiologically relevant measure of digestible protein. While it is not necessarily realistic to expect absolute agreement between *in vitro* and *in vivo* observations, *in vitro* assays should not be ruled out as useful tools. They can be used to rank food proteins according to their protein digestibility, to further our understanding of protein structure under digestion conditions⁽⁵³⁾, and with further refinement could be useful for the prediction of *in vivo* nutritive value. The development of multiple regression equations including *in vitro* digestibility measures combined with chemical constituent measures for individual foods has been recommended⁽⁶⁾. In addition, *in vitro* digestion techniques have an important role to play in extending our understanding of the release of amino acids and peptides during digestion and the influence protein structure plays on dietary protein quality⁽⁵⁴⁾.

Evaluation and validation with *in vivo* assays

Many of the studies evaluating *in vitro* protein digestibility assays have compared the *in vitro* data with *in vivo* faecal digestibility. The influence of the microflora in the large

intestine however, does not make this comparison useful. The ileal method for determining crude protein digestibility is more accurate⁽⁵⁵⁾ than the faecal approach, and it is more relevant to *in vitro* measures given that these methods do not simulate microbial digestion in the large intestine⁽⁵⁶⁾. It has been recommended previously that protein sources that do not contain fibre or anti-nutritional factors should be evaluated against true ileal protein digestibility⁽¹⁹⁾, while plant proteins including grains should be evaluated against real ileal protein digestibility⁽⁶⁾. Further details of these *in vivo* assays are given elsewhere in this supplement. There are only a few studies that have used these recommended comparisons and it is therefore difficult to properly evaluate the accuracy of current *in vitro* assays.

The determination of true or real ileal amino acid and nitrogen digestibility requires access to the terminal ileum, which is usually obtained via surgery, as well as a specific feeding or labelling technique to measure endogenous losses. The determination of *in vivo* protein and amino acid digestibility in humans is generally not possible so researchers use animal models. The pig^(57,58) and the rat^(59,60) have been found to be suitable animal models for humans. There is very little published information on ileal amino acid digestibility determined for humans directly⁽⁶¹⁾, making the evaluation of *in vitro* digestibility data even more difficult. One of the few studies to compare human digestibility (albeit faecal digestibility) and *in vitro* digestibility values on the same foods is that of Bodwell *et al.*⁽⁵⁹⁾. They found there was no significant correlation between *in vitro*, human and rat digestibilities when all the proteins were considered together. However, when the data were analysed with the food proteins identified separately as of animal or plant origin the correlations were high ($r > 0.90$).

There have been mixed results in studies comparing *in vitro* and *in vivo* ileal digestibilities in pigs on the same protein sources ranging from no significant correlation⁽⁶²⁾, low correlation⁽⁶³⁾, to good correlations^(64,65). A comprehensive study of a wide range of feedstuffs⁽⁶⁶⁾ used standardised true ileal nitrogen digestibility determined in the growing pig and a two-step pepsin/pancreatin *in vitro* digestion. High correlations were obtained within feedstuffs when the prediction equations also included terms for specific chemical components of the foods. As would be expected, true ileal digestibility gave better correlations than when apparent ileal digestibility was used. The authors concluded that due to differences in endogenous protein loss induced by the different foods a single prediction equation based on the *in vitro* assay would not be appropriate.

Methodology needs to be standardised across research organisations^(67,68) and reference proteins should be included in every assay for comparison across studies. *In vivo* digestibility is influenced by the experimental conditions, and the way digestibility is calculated such as the presence of endogenous protein, and methods of endogenous loss correction^(69,70). It is now widely accepted that protein and amino acid digestibility must be determined using accurate measurements of dietary intake and terminal ileal digesta flow corrected for basal endogenous protein loss to give 'true' digestibility values.



However, some foods also contain components (dietary fibre, anti-nutritional factors) that induce higher endogenous protein losses than the basal losses measured with purified highly digestible protein-free diets. There have been only a few studies^(7,71) that have attempted standardisation of methodology and more need to be published⁽⁷⁾, and correlations determined both within and across food proteins.

Conclusions and recommendations

Simple *in vitro* digestion methods have the potential to give useful measures of *in vivo* amino acid and protein digestibility for humans. An *in vitro* method to measure the extent of digestion of protein must be accurate, rapid, cheap, simple, robust, adaptable and relevant to the processes of digestion, absorption, and metabolism. The more complex *in vitro* methods including the computer controlled *in vitro* models of the digestive tract that are attempting to more closely mimic the processes of digestion and absorption may be too expensive and time consuming for the rapid and routine analysis of food protein quality but are useful tools in expanding our understanding of digestion processes. All these methods require independent validation with *in vivo* data from the target species or an acceptable animal model.

The literature contains many papers describing the development of *in vitro* protein digestibility assays, but all too often no validation results are given or if they are, an inappropriate *in vivo* digestibility baseline has been used. Very few studies have included a full developmental process where the sensitivity of important assay variables is tested and then the variables optimised. Repeatability is seldom examined or at least reported. Promising methods have often been published and applied, only to fall into obscurity over time, presumably because they do not provide consistently accurate results.

Over the last few years, the application of *in vitro* digestibility assays is becoming common, particularly in food science research to examine effects of food physical and chemical structures on nutrient digestibility. There is a pressing need for the thorough development of an agreed (i.e. standardised) protein digestibility assay with application to human foods. Such an assay should be fully tested independently of the developer in a number of different laboratories around the world, and should be evaluated against relevant observations of *in vivo* digestibility.

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