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 vitro measure of digestibility. For protein sources devoid of anti-nutritional factors or plant fibre, true ileal digestibility is the recommended in vitro baseline, while for plant proteins the recommended in vitro 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 impossible12 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 reviewed2–5. In vitro protein digestibility assays and their validation with in vivo measures have been reviewed by Moughan6. 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 rennin2. 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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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 tryptic 

of 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, trypsin, or intestinal fluid from pigs. 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. Good correlations were found with in vitro digestibility (rat true faecal) particularly when the protein sources were analysed by electrophoresis, immunochemical and mass spectrometry techniques. 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. The components of some food materials, however, interfere with the pH drop due to their buffering capacity.

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 vitro protein digestibility, was reproducible, and gave high correlations with in vivo (rat faecal) digestibility for highly digestible animal protein sources.

The immobilised digestive enzyme assay (IDEA) system 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 with rat faecal protein digestibility for a range of foods.

Gauthier et al. developed an in vitro digestion under constant dialysis (molecular weight cut off 1000 Daltons) with a 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 vitro protein digestibility, was reproducible, and gave high correlations with in vivo (rat faecal) digestibility for highly digestible animal protein sources.

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In 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 and has also been used in pharmaceutical studies. Model stomach and small intestine 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 aspects of food digestion and absorption.
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\(^{(69)}\). Their validity for predicting the in vitro 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; standardizing 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\(^{(66)}\). 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\(^{(60)}\). 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\(^{(61)}\). 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\(^{(60)}\). 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 vitro 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 vivo, 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\(^{(52)}\), 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 vitro 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\(^{(66,70)}\). 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 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 vitro* 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. Repetatability 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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