In vitro techniques to estimate amino acid digestibility

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Abstract

When a food is processed or stored, amino acids can react with a number of chemical entities to produce new compounds that are often nutritionally unavailable to the consumer. Lysine is an important indispensable amino acid, and describing the lysine content of a food or feedstuff provides useful information about nutritional value. Chemical assays have thus been developed to allow determination of unaltered or reactive lysine. The in vitro procedures include three chemical procedures: the furosine procedure, the reactive lysine procedure and KOH solubility.

Keywords: amino acid digestibility, lysine, Maillard reaction

Introduction

The primary function of dietary protein is to furnish amino acids for the synthesis of tissue proteins.

When predicting the nutritional quality of foods, information on the digestibility of the various nutrients is of utmost importance. However, *in vivo* determinations are time consuming and costly, which is why much effort has been devoted to the development of *in vitro* procedures.

An important and frequently observed effect of food processing is the reduction of protein nutritive quality.

The loss in digestibility is due to the denaturation of the protein and reduction in amino acid availability by cross-linking, racemization, degradation and formation of complexes with sugars [1]. Therefore, when attempting to estimate protein quality, one of the first factors that must be evaluated is the digestibility.

Technological processes, applied to cereals, can cause modifications in their composition.

In heat-processed proteins, some amino acids may be unavailable owing to interactions among themselves or with other components of the food or feed, or to alteration of the amino acid molecule itself [2]. Lysine and methionine are the amino acids most subject to such changes.

The heating process induces one of the most important modifications, the Maillard reaction, which involves amino acids (mainly lysine) and reducing carbohydrates [3] and can produce a loss of nutritive value [4,5].

The Maillard reaction is a general term used to describe a complex series of reactions between reactive carbonyl groups, such as those of reducing sugars, and free amino groups of proteins. Lysine is the most important carrier of free amino groups in proteins, in the form of ε-amino group, and therefore is the most significant amino acid participant in the Maillard reaction. Beside lysine, arginine, tryptophane, and histidine are also carriers of free amino groups.

Studying the effect of the Maillard reaction products on protein digestion, Oste *et al.* determined that low-molecular-weight compounds developed in the reaction of glucose and lysine inhibited N-amino peptidase [6]. This inhibition resulted in

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reduced protein absorption in the digestive tract.

Despite more than 90 years research work in this area, the molecular mechanisms are still not well understood, although an increasing number of the Maillard reaction products have been identified [7]. Low-molecular-weight products of the Maillard reaction have an exceptionally important role in the formation of flavour, aroma, colour and texture in thermally treated foods. The Maillard reaction partially proceeds during storage. Melanoid products are also formed in the reaction of amino acids or proteins with oxidized lipids. These products are of the similar structure as those developed in the reaction with reducing sugars. Finot (1990) offered a useful summary of physiological and pharmacological effects of the Maillard reaction products [8]. Briefly, these effects include: (a) inhibition of processes such as growth, protein and carbohydrate digestion, amino acid absorption and activity of intestinal enzymes including amino peptidases, proteases, and saccharidases, and pancreatic enzymes such as chymotrypsin; (b) induction of cellular changes in the kidneys, liver, and stomach cecum; (c) adverse effects on mineral metabolism (Ca, Mg, Cu and Zn); (d) variable effects on allergic response and cholesterol metabolism. However, the Maillard reaction products showed not only adverse effects, but also anti-oxidative effects, as well as antimutagenic, antibiotic and antiallergenic effects [9,10,11]. Various Maillard reaction products, obtained under strictly controlled conditions, are used as commercial food additives; e.g., as food aromas and antioxidants.

The Maillard reaction is the major non-enzymatic sequence of reactions that causes protein to become nutritionally unavailable when heated. The amino acid most vulnerable to heat damage is lysine [12].

The ε-amino group of protein-bound lysine can react with glucose, maltose or lactose to form Amadori products, which are not susceptible to attack by proteolytic enzymes during digestion [13]. Loss of available lysine, which is the most negative nutritional consequence of the Maillard reaction, is particularly significant in cereals, in which this amino acid is limiting [14].

Only amino acids that can be incorporated into tissue proteins are bioavailable. Bioavailability is defined as the proportion of dietary amino acids that are absorbed in a chemical form that is suitable for protein synthesis [15,16].

The term "amino acid digestibility" does not refer to the digestion of amino acids – it only refers to the digestion of the peptide bonds connecting the amino acids in a dietary protein [17].

Because the nutritional quality of a protein is related both to its amino acid content and the capacity of digestive enzymes to liberate them, methods using digestion enzymes have been tried to estimate the nutritive value of proteins. Until the present, an extensive methodology has been developed.

In vitro assays can be divided into five groups namely: a) Chemical assays; b) Enzymatic assays; c) Microbiological assays; d) Near infrared (NIR) spectroscopy; e) Immunological assays

Many biological methods have also been used. Enzymatic methods have been developed by Mauron *et al*. [18] and Sheffner *et al*. [19]. Schweigert and Guthneck [20] and Guthneck *et al*. [21] developed a method based on the growth of the protein depleted adult rat. Kuiken and Lyman [22] and Kuiken [23] measured the amount of ingested lysine excreted in the feces of rats fed different foodstuffs. Gupta *et al*. [24] used the rate of growth of the young rat over a 2-week period as the basis for measuring lysine availability. Ousterhaut *et al*. [25] estimated the availability of various essential amino acids in proteins from the growth of chicks fed a basal diet containing crystalline forms of all of the essential amino acids except the one being assayed, with the protein under test as the only source of the amino acid.

The evaluation of available lysine by the biological analysis of growing rats or chicks is a laborious process with a high coefficient of variation and has been replaced by faster methods, such as evaluation of plasma amino acids, *in vitro* enzymatic determination, microbiological measurement and mainly chemical methods [26].

The enzymatic procedures are: the one-step pepsin procedure, and the two-step pepsin-pancreatin procedure. The one-step pepsin procedure involves the incubation of a sample at 37°C in pepsin for 8 or 18 hours at pH 2.5 [27]. The sample is filtrated after incubation and the undigestible portion of the protein is believed to be present in the filtrate. By analyzing this portion and relating it to the total protein content in the sample, the disappearance of protein is calculated and assumed to be equal to the digestibility of the protein.

The two-step pepsin-pancreatin procedure involves sample incubation at 39°C at pH 2 with pepsin followed by the incubation with pancreatin (a mixture of pancreatic proteolytic enzymes) at pH 6.8. Following the incubation, all samples are filtrated and washed in ethanol and acetone and dehydrated. The filtrate is then analyzed for the concentration of crude protein. The procedure was initially developed for predicting amino acid digestibility in mixed diets [28]. Alternatives to this procedure, including longer incubation times, were later introduced to predict amino acid digestibility in meat and bone meal [29].

Chemical methods for assessing the digestibility of amino acids have been almost invariably associated with lysine [30] for two reasons:

a) Lysine is often the factor limiting the protein quality of mixed diets for man or animals.
b) Its ε-amino group is highly sensitive to the Maillard reaction during heat treatment.

In vitro techniques to determine lysine digestibility

Lysine is an important indispensable amino acid, and describing the lysine content of a food or feedstuff provides useful information about nutritional value.

L-lysine is an essential amino acid, which means that it is necessary for human health but cannot be made by the body. For this reason, lysine must be obtained from food. Lysine is important for proper growth, and it plays an essential role in the production of carnitine, a nutrient responsible for converting fatty acids into energy and helping to lower cholesterol. Lysine appears to help the body absorb and conserve calcium, and it plays an important role in the formation of collagen, a substance important for bones and connective tissues including skin, tendon, and cartilage.

If there is too little lysine in the diet, kidney stones and other health related problems may develop including fatigue, nausea, dizziness, loss of appetite, agitation, bloodshot eyes, slow growth, anemia, and reproductive disorders. It is extremely rare, however, to obtain insufficient amounts of lysine through the diet. Generally, only vegetarians who follow a macrobiotic diet and certain athletes involved in frequent vigorous exercise are at risk for lysine deficiency. For vegetarians, legumes (beans, peas, and lentils) are the best sources of lysine.

The nutritive value of a food protein depends not only on its content of essential amino acids but also on physiological availability. Amino acids are unavailable if they are in regions of a protein protected (chemically or physically) from action of proteolytic enzymes or if they are linked to other chemical moieties through bonds not readily broken by digestion.

Cross-linking is probably the most important chemical mechanism restricting biological utilization. Because of its ε-amino group, lysine is particularly susceptible to side reaction and cross-linking making it unavailable. For more

than 15 years the chemical and biological availabilities of lysine in processed foods and feedstuffs have been studied extensively. Lysine is especially important in wheat products because their lysine content is low to begin with and is liable to further degradation by moderate to severe heat treatments that some of these products receive.

Lysine becomes secondarily bound (nutritionally unavailable) as a result of chemical reaction of its ε-amino group including the following:

First, lysine may be buried in a protein matrix in a particular sequence, or conformation, which is slow to hydrolyze or is not hydrolyzed at all by animal proteases; such lysine may or may not appear as chemically available by hydrolysis methods and yet be totally unavailable nutritionally.

Second, lysine can be cross-linked to an aspartyl or glutamyl residue on another protein or in the same protein molecule.

Harding and Rogers [31] have isolated such a cross-link from hair and Asquith and Otteburn [32] and Holt and Milligan [33] demonstrated the presence of such cross-links in heated keratin proteins. Although the bond looks like a "normal" peptide bond it is not hydrolyzed by gut proteases. The aspartyl- or glutamyl-lysine remains after proteolysis and appears nutritionally unusable. The aspartyl- or glutamyl-lysine link is broken by acid hydrolysis, which yields lysine and aspartic or glutamic acid. Another reaction of lysine is cross-linking with dehydroalanine residues. Bohak [34] and Patchornik and Sokolovsky [35] reported that dehydroalanine is formed by heating serine or cysteine under alkaline conditions.

When a food is processed or stored, amino acids can react with a number of chemical entities to produce new compounds that are often nutritionally unavailable to the consumer. During acid hydrolysis used in amino acid analysis, some of these compounds revert back to the parent amino acid, leading to errors in estimates of both the amino acid content of foods and amino acid digestibility. This is a particular concern for the amino acid lysine in damaged food proteins. Chemical assays have thus been developed to allow determination of unaltered or reactive lysine. However, there is evidence that, in damaged food proteins, not all of the reactive lysine is released during digestion and absorbed.

Lysine is involved in the browning reaction, or caramelization, in foods such as pastries, doughnuts, cookies, and cereals. In this process, lysine and sugar become linked together in a way that makes lysine difficult for the body to absorb. As a result, a diet high in cereals and baked goods, especially those that contain a lot of simple sugars, can result in low lysine intake.

The *in vitro* procedures to measure bioavailable lysine include three chemical procedures: the furosine procedure, the reactive lysine procedure and KOH solubility.

The use of *in vitro* techniques to measure bioavailable amino acids is attractive because it is relatively simple, rapid, reproductible and it avoids the use of animals.

Furosine concentration procedure

During heat treatment of proteins, the ε-amino group of free lysine and protein-bound lysine may react with reducing sugars in the Maillard reaction [36,37]. This reaction generates both early and late Maillard products. The early Maillard products are structurally altered lysine derivatives that are called Amadori compounds, deoxy-ketosyl derivatives, or blocked lysine, while the late Maillard products are called melanoidins. The melanoidins will not be identified in the regular analysis for amino acids and will only result in lower lysine to crude protein ratios in the product. The calculation of the lysine to crude protein ratio gives an indication of the extent of conversion of lysine to melanoidins, and therefore, of the degree of heat damage that has occurred. The melanoidins do not interfere with the

normal analysis for lysine and have no influence on the digestibility values that are calculated – they only result in lower concentrations of lysine in the sample, and therefore, in lower quantities of lysine being absorbed.

The Amadori compounds (early Maillard products), on the other hand, interfere with the amino acid analysis and give inaccurate lysine concentrations in the sample being analyzed. These products are derived from the reaction of lysine with lactose, fructose, aspartate, or glutamate and the resulting derivatives are called lactulosyllysine, fructosyllysine, aspartyllysine and glutamyllysine, respectively [10]. The lysine that is bound in these compounds is called "blocked lysine" and is biologically unavailable because it is resistant to gastrointestinal enzymatic breakdown [38]. However, during acid hydrolysis, 40 to 50% of the blocked lysine is released as lysine [39] while the rest is released as furosine and pyridosine. The resulting ileal digestibility values will, therefore, overestimate the digestible lysine content in the sample, but will underestimate the digestibility coefficient of the un-blocked lysine [40]. Because lysine, furosine and pyridosine are released from the blocked lysine in a constant ratio, the total amount of blocked lysine may be calculated by analyzing either the furosine or the pyridosine concentration in the sample. Furosine is easily analyzed using high performance liquid chromatography (HPLC). Subsequently, the concentration of blocked lysine has been calculated assuming that the furosine concentration equals 20% of total blocked lysine. The correlation between the calculated blocked lysine and the *in vivo* standardized digestibility of lysine in Distillers Dried Grains with Solubles (DDGS) was calculated at 0.71. This procedure seems to hold some promise as an accurate method to estimate the digestibility of lysine in DDGS. The digestibility of amino acids other than lysine can, however, not be predicted using this procedure.

Reactive lysine procedure

While the furosine procedure can be used to estimate the quantity of blocked lysine in heat damaged feed samples, the reactive lysine method estimates the amount of lysine that is not bound to any food component in a manner that hinders lysine digestibility or availability. This lysine is called the reactive lysine and is available for absorption and utilization by the organism.

For foodstuffs that are not heated, the total lysine content in the sample is not contaminated with Amadori compounds and all the analyzed lysine in the sample is expected to be available for absorption and utilization. In such a food ingredient the reactive lysine would be expected to be equal to the total analyzed concentration of lysine. However, in heat damaged proteins, a portion of the analyzed lysine may consist of Amadori products and introduce errors in the calculation of digestible lysine as discussed above [41]. In the reactive lysine procedure, the quantity of lysine that is not bound in the Amadori compounds is quantified.

Because this is the only portion of the analyzed lysine that is available for absorption, a quantification of this portion will be directly related to the digestible quantities of lysine [42]. There are at least three methods available to determine the reactive lysine content of a food or feed sample. All three methods involve the chemical conversion of the reactive lysine in the food ingredient to a different chemical compound: dinitrophenyl lysine, trinitrophenyl lysine, or homoarginine. For samples that may contain starch, the conversion of reactive lysine to homoarginine is believed to be the most accurate. This procedure involves the reaction of the food sample with O-methyl-isourea, which will convert the reactive lysine (but not the blocked lysine) to homoarginine. By measuring the conversion of total lysine to homoarginine, the quantity of reactive lysine can be estimated. Because the quantity of reactive lysine is supposed to be directly related to

the digestibility of lysine, the conversion of lysine to homoarginine is believed to be correlated to the digestibility of lysine. Preliminary work has resulted in a medium correlation to the standardized ileal digestibility of lysine in samples of DDGS. However, there are several methodological aspects of this procedure that still need to be researched and it is possible that the reactive lysine procedure may be refined and eventually yield better correlations to *in vivo* data.

Probably one of the most widely used methods to measure protein quality is that developed by Carpenter and his colleagues, using 2,4-dinitrofluorobenzene (DNFB) to determine available lysine. In proteins only the N-terminal amino acids and the ε-amino group of lysine are considered to react with DNFB. Undamaged lysine contains ε-amino groups that can bind with the reagent (DNFB) to form a complex that can be measured spectrophotometrically [43,44].

Carpenter (1960) used selective solvent extract to separate ε-dinitrophenylated lysine from the reaction mixture and methyl chloroformate to overcome interference caused by arginine.

The principle of the assay is that the ε-amino group present in the lysine residues in a protein may react in some way (e.g. with an aldehyde group such as is present in sugars or with a carboxyl group from other amino acid residues) preventing it from binding with 2,4–dinitrofluorobenzene. The biological hypothesis is that only lysine molecules with free ε-amino groups (i.e. those reacting with 2,4–dinitrofluorobenzene) are nutritionally available to man and animal [45]. There are evidences suggesting that lysine residues with blocked ε-amino groups are either not released by the digestive enzymes, or if they are released, they are not absorbed from the gastrointestinal tract. The data generated from this method were found to be well correlated to that of the growth assays in chicks [46,47].

The DNFB method is time consuming and it causes allergies especially in the female operators as experienced in the analytical laboratories.

Kakade and Liener developed alternatives to overcome problems with the DNFB method and used 2,4,6-trinitrobenzene-sulfonic acid (TNBS) [50]. Excellent results were obtained from this method from e.g. fish meal, but processed soy analysis was not possible since a milky supernatant was produced, which lead to negative values in the spectrophotometric determination. This problem was overcome by applying high-speed centrifugation (17000 rpm).

Alternative methods have been developed for chemically measuring the reactive lysine [48,49]. These include the use of O-methylisourea (OMIU) or the guanidination reaction [51], furosine [52], dye-binding with sulfonated dyes such as acid orange 12 [53], and total lysine determination after reduction with borohydride ($NaBH_4$) treatment [52].

In comparing all these methods of measuring lysine in heat-damaged proteins, Hurrell and Carpenter found that the DNFB and OMIU procedures were the most suitable methods for heat damaged products [53].

KOH – solubility

A chemical assay, which is widely used, is the KOH-method to measure protein solubility, especially as an indicator of over-processed oilseed meals [54]. The protein solubility in KOH has been shown to be a sensitive index of over-processing of soybean meal [55,56,57]. By far, the most important process encountered with soya products is heat treatment. Defatted soya flour and grits, full-fat soya flour and grits, soyamilk, soyamilk curd and soya protein concentrate, among others, include a heat treatment step in their production.

Cooked (denatured) proteins are less soluble than raw proteins. Solubility therefore gives an indication of the extent of cooking.

Lower KOH value means lesser digestible lysine due to the Maillard reaction. Higher KOH value means more digestible amino

acids but lesser breakdown of trypsin inhibitors present in soybean, leading in less digestion and absorption of amino acids [39,58].

The procedure involves the incubation of a sample with a 0.2% KOH solution for 20 min at room temperature. Following this incubation, the sample is centrifuged and the supernatant is analyzed for the nitrogen concentration.

Dale and Araba [59] investigated the KOH-solubility method. They observed that the protein solubility below approximately 70% had a poor growth effect on chickens fed diets containing soybean meals as the major source of essential amino acids. A value of above 85% indicates that soybeans could be under processed.

KOH solubility is not a suitable method for assessing the protein quality of an alcohol extracted soy protein concentrate, which normally has low KOH protein solubility, but its low protein solubility is a result of alcohol denaturation of the protein rather than overheating.

Acknowledgements

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