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# Proteolytic Activity, Amino Acid Composition and Protein Quality of Germinating Fenugreek Seeds (*Trigonella foenum graecum* L.)

Ahmed Rafik El-Mahdy

Food Science Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

&

Laila A. El-Sebaiy

Food Technology Department, Kafr El-Sheikh, Faculty of Agriculture, Tanta University, Tanta, Egypt

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*ABSTRACT*

*This study determined the changes in proteolytic activity and trypsin inhibitor, due to germination for 120 h, and their relationships to amino acid content and protein quality. The activities of cas-ase, Hb-ase, and BAPA-ase, as well as the trypsin inhibitor of the fenugreek, increased as germination proceeded. Glutamic and aspartic acids were the richest amino acids in fenugreek seeds, followed by leucine, arginine, lysine and proline. After germination, the contents of aspartic acid, phenylalanine, tyrosine, threonine, tryptophan and valine increased, while those of glutamic acid, proline and four of the essential amino acids decreased. All the free amino acids increased after germination, while glycine decreased. Cystine, tryptophan and proline originated in a free form after germination. The chemical score, based on the essential amino acid pattern of whole egg protein, for fenugreek seeds, showed a marked decrease (21%) after germination. The* in vitro *pepsin, followed by*

*pancreatin digestion, showed a slight increase for the germinated seeds. Germination of seeds lowered the C-PER, a result which indicates that the protein quality decreased.*

## INTRODUCTION

Fenugreek, *Trigonella foenum graecum* L., is one of the most widespread legumes in certain countries of the Mediterranean region, of Europe, the Near East, India, Argentina and Egypt (Shankaracharya & Natarajan, 1972). Fenugreek seeds are used as a spice, as a vegetable for human consumption, as forage for cattle and, to a limited extent, for medical purposes.

The protein content of the seeds ranges from 20 % to 30 % (Kolousek & Coulson, 1955; Shankaracharya & Natarajan, 1973; El-Madfa, 1975; El-Mahdy & El-Sabaiy, 1982). The amino acid composition shows that the proteins of fenugreek are very similar in their composition to other leguminous proteins such as soybean (Kolousek & Coulson, 1955; El-Madfa, 1975; Jamalian & Pellett, 1968; Sauvaire *et al.*, 1976).

Fenugreek is believed to be a good supplement for cereals because of its high lysine and tryptophan contents (Talwalkar & Patel, 1970; El-Madfa, 1975). Studies by Irving & Fontaine (1945) and Beevers (1968) on germinated legumes revealed marked changes in the nutrients involved in the breakdown of seed reserves and their utilization by the growing parts, while Young & Varner (1959) and Henshall & Goodwin (1964) demonstrated only slight changes in proteolytic activity with germination. Hegazi (1974) reported that the increased proteolytic activity is accompanied by interconversion and utilization of amino acids in the production of new nitrogenous compounds. This leads to various changes in the amino acid content, which may affect (positively or negatively) the nutritive value of the protein (Hegazi, 1974; Fordham *et al.*, 1975; Bates & Krapp, 1977; El-Nahry *et al.*, 1977; Chen & Thacker, 1978).

El-Mahdy & El-Sebaiy (1982) found that, after the fourth day of fenugreek germination, there was a decrease of protein nitrogen and a marked increase of both non-protein nitrogen and free amino acid nitrogen.

No data were found in the literature concerning the effect of germination on the activity of the proteolytic enzymes and amino acids of fenugreek seeds. The experimental work described in this paper was therefore carried out to determine the changes in the proteolytic activity

of fenugreek seeds, as well as in the trypsin inhibitor, due to germination and their relationship to amino acid content and protein quality.

## MATERIALS AND METHODS

### Materials

*Seed type and germination procedure*
Fenugreek seeds (*Trigonella foenum graecum* L.), Giza II variety, were purchased from an Egyptian market at Alexandria. The seeds were cleaned, surface sterilized by soaking in ethanol (95%) for 1 min, washed with distilled water, then soaked in five volumes of distilled water for 6 h at room temperature (26–30 °C). The water was then removed and the germination begun on a screen in the dark for 120 h. The seeds were washed twice every 12 h with distilled water. The seedlings were dried overnight at 40 °C in a fan assisted oven. Germination for the assay of the proteolytic and antitryptic activities was carried out by the same method using 1 g of seeds placed in a Gooch crucible. After germination, the germinated seeds were taken fresh for the assay of proteolytic and anti-trypsin activities.

*Sample preparation for analyses*
The ungerminated (UGS) and germinated dried seeds (GS) were ground in a laboratory Wiley mill to pass through a 40 mesh sieve. The ground samples were stored at 5 °C until analysed. All samples were analysed in triplicate.

### Methods

Moisture and total nitrogen (TN) were determined by the method of the Association of Official Analytical Chemists (1975). The microKjeldahl method was used for TN estimation and the crude protein was calculated by multiplying total nitrogen by a factor of 6·25.

*Determination of total free amino acid nitrogen*
The total free amino acids (TFAA) were determined by means of the ninhydrin colorimetric method of Rosen (1957). The germinated and ungerminated samples were extracted by shaking for 1 h with 0·8M trichloroacetic acid (TCA) solution. Leucine was used as a standard amino acid to make up a calibration curve.

*True protein determination*

The total protein soluble in alkali was estimated according to the method of Lowry *et al.* (1951) after being extracted with 0·1M sodium hydroxide. Protein concentration was expressed as milligrams per gram of dry sample.

*Extraction of free amino acids*

A 1-g sample was extracted with hot ethanol, 70% (w/v), by homogenization (Unipan laboratory aid 309), followed by centrifugation for 5 min at 3500 *g*. The supernatant was evaporated under vacuum (Gallenkamp thin film evaporator) and the dry residues were dissolved in 0·2M citrate buffer, pH 2·2.

*Amino acid analyses*

Acid protein hydrolysates were prepared for the analyses of all amino acids except tryptophan. Fifty millilitres of 6M hydrochloric acid (HCl) containing 25 $\mu$l of mercaptoethanol were added to 200–300 mg of sample (7–12 mg total nitrogen) and refluxed under a stream of nitrogen at 110°C ± 5°C for 24 h. The hydrolysate was then cooled to room temperature and filtered through Whatman No. 52 filter paper to remove any visible humin sediments. The humin precipitate was washed with distilled water and the volume of the filtrate made up to 100 ml. A suitable volume of the filtrate (20 ml) was placed in a desiccator over potassium hydroxide and beside phosphorus pentoxide. Hydrochloridic acid removal and drying of hydrolysate were carried out by evacuating the desiccator overnight to approximately 10 mm Hg. The dry residues were dissolved in 25 ml of 0·2M citrate buffer.

The alkaline hydrolysate was prepared for tryptophan determination. A sample was refluxed with 0·443M solution of barium hydroxide octahydrate for 20 h as described by Block *et al.* (1958).

The individual total and free amino acids, except for tryptophan, were estimated by the method described by Spackman *et al.* (1958) using a Beckman 119CL amino acid analyser. The response of the amino acid analyser was checked by analysing a standard mixture of seventeen commonly occurring amino acids in protein hydrolysates and ammonia and the recoveries obtained were used to calculate the amounts of the amino acids in the various samples.

Tryptophan was estimated colorimetrically using the *p*-dimethyl-aminobenzaldehyde reagent of Miller (1967).

*Extraction and assay for proteolytic enzymes*

The proteolytic enzymes were extracted from fenugreek samples (1 g) using the method of Bhatty (1969). The samples were homogenized (Waring blender) for 4 min with 40 ml 0·15M saline containing 0·5 % (w/v) L. cysteine HCl, pH 7·0. The volume of the homogenate was adjusted to 100 ml with saline, strained through two layers of cheesecloth to remove debris and centrifuged at 3000 *g* for 20 min. Aliquots (0·1 ml) were taken from each extract for the assay of proteolytic activity. Two natural substrates—casein and haemoglobin—were used as described by Bhatty (1969) to assay the cas-ase and Hb-ase activities, respectively. The digestion products of the two enzymes were estimated using the Lowry *et al.* (1951) method. A synthetic substrate—*N*-α-benzoyl-DL-arginine-*p*-nitroaniline hydrochloride (BAPA)—was used to determine the BAPA-ase activity using the method of Kakade *et al.* (1969). The liberated *p*-nitroaniline was determined colorimetrically at 410 nm. Cas-ase and Hb-ase activities were expressed as units per millilitre of extract. The unit is defined as the quantity of enzyme which gives, at the assay conditions, 1 μM of liberated tyrosine (Greenberg, 1955). BAPA-ase activity was expressed as absorbance units, for a 1 cm light path, at 410 nm per millilitre of extract under the assay conditions (Kakade *et al.*, 1969).

*Trypsin inhibitor activity*

Trypsin inhibitor activity of germinated and ungerminated fenugreek samples was determined using 'BAPA' as a substrate (Greenberg, 1955). The fenugreek extract was prepared by homogenization of the sample in a known volume of distilled water, the pH of the suspension being adjusted to 7·6. The suspension was shaken for 1 h and centrifuged at 4000 *g* for 20 min. One millilitre of the extract was diluted to 50 ml for protein estimation (Lowry *et al.*, 1951). The trypsin inhibitor units were expressed in terms of the trypsin units inhibited per millilitre of extract (Kunitz, 1947). The trypsin unit was defined as an increase of 0·01 absorbance units, at 410 nm, of the reaction mixture under the experimental conditions (Kakade *et al.*, 1969).

*Protein quality evaluation*

The calculated protein efficiency ratio (C-PER) is an *in-vitro* assay for the prediction of the PER of food proteins. PER was assayed and computed according to the method of Hsu *et al.* (1978). This method utilizes the data obtained from amino acid analyses of samples, as well as casein and the *in*

*vitro* protein digestibility, for samples and a reference protein—casein—to predict the C-PER of the samples.

The *in vitro* digestibilities of the fenugreek protein and casein were determined using pepsin followed by pancreatin. The digests were prepared as described by Akeson & Stahman (1964). The protein digestibility was expressed as a percentage of digested protein nitrogen referred to the total nitrogen. The amino acid and chemical scores were calculated using the essential amino acid profile (EAA) and the equations given by Pellet & Young (1980).

## RESULTS AND DISCUSSION

### Germination and soluble protein

Characteristically, it was found (Fig. 1) that, as germination progressed, there was a depletion of the soluble protein (SP) with an accumulation of

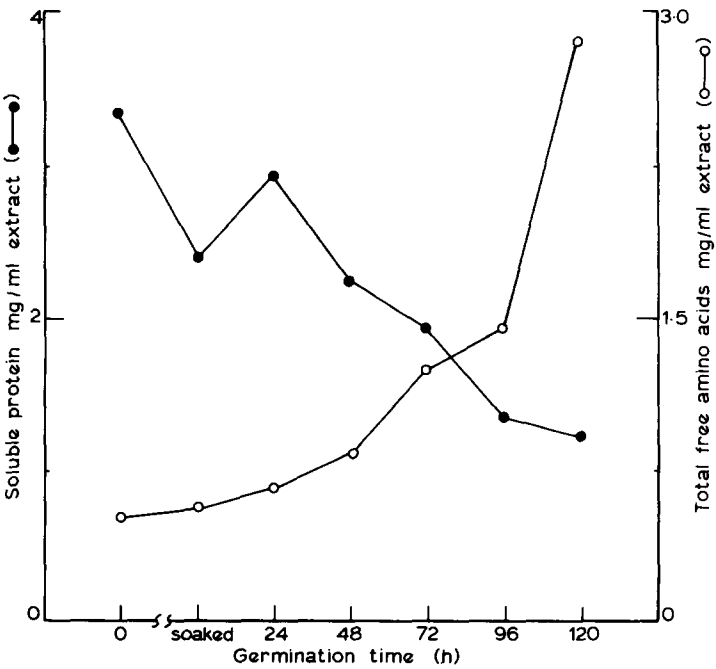


**Fig. 1.** Effect of germination time on the concentration of protein and total free amino acids of fenugreek seeds.

total free amino acids (TFAA). The depletion in the SP content of the germinating fenugreek appeared to indicate *in situ* utilization of soluble protein by the germinating seeds. As shown in Fig. 1, the decline of SP was most rapid between the third and fifth days of germination. These observations are consistent with the suggestions of Ingle *et al.* (1964), Deshmushi & Sohonic (1966) and Hegazi (1974) that cotyledonary proteins are hydrolysed to amino acids before they are used for the formation of proteins for the newly developing parts. However, complete utilization of the free amino acids liberated after germination does not occur, since there is a net increase of the TFAA. These results are in agreement with those reported by El-Mahdy & El-Sebaiy (1982) who found that, after 76 hours' germination, the free amino acid nitrogen of fenugreek seeds increased by 298% while the protein nitrogen decreased by 27%.

**Effect of germination on proteolytic and anti-tryptic activities**

The proteolytic activity of fenugreek seed extracts during germination was examined against two natural substrates—casein and haemoglobin—and another synthetic substrate, *N*-α-benzoyl-DL-arginine-*p*-nitroaniline (BAPA). Figure 2 shows the estimated cas-ase, Hb-ase and BAPA-ase activities. It was possible to demonstrate that, in spite of the variation in their pH optima (cas-ase and Hb-ase, 7·6; BAPA-ase, 8·6), the development of these enzymes in germinating fenugreek was similar. Initially, the activities were low but increased as germination proceeded. The release of digestion products from the casein and haemoglobin substrates was initially slow, being maximal by the fifth day of germination for cas-ase and Hb-ase. This might indicate that the enzymes being assayed were non-specific exo-peptidases and the low initial rate of digestion might be related to the lack of the available free ends in the protein molecules.

Since BAPA does not possess free terminal α-amino or carboxyl groups, it is most likely that this substrate is hydrolysed by endo-peptidases. It is clear that the enzymatic release of *p*-nitroaniline from BAPA increased gradually during the course of germination. The observed increase in proteolytic activity, as germination proceeded, agrees with the findings of Irving & Fontaine (1945) for germinating peanuts and of Beevers (1968) for germinating peas, while the BAPA-ase

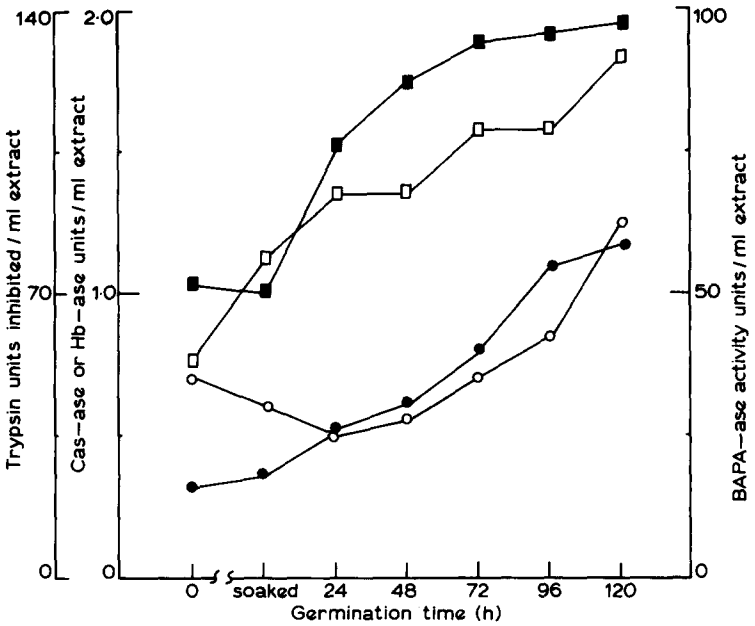


**Fig. 2.** Effect of germination on the proteolytic and anti-tryptic activities of fenugreek seeds. ○, Cas-ase; □, BAPA-ase; ●, Hb-ase; ■, anti-tryptic activity.

activity present is at variance with the observations of Beevers (1968). He found that BAPA-ase activity fell rapidly during the germination of peas.

As reported by El-Mahdy & El-Sebaiy (1982), the activity of trypsin inhibitor per gram of ungerminated fenugreek is equal to the amount present in soybean (*Glycine max*) and twice the amount in faba beans (*Vicia faba*). The results of anti-tryptic activity are shown in Fig. 2. The trypsin inhibitor activity of the fenugreek seeds increased as germination proceeded, to reach 1·9-fold its original value. This finding is contrary to that reported by Hobday *et al.* (1973) for peas and by El-Hag *et al.* (1978), for red kidney beans.

## Effect of germination on the individual, total and free amino acids

The nutritive value of a dietary protein is dependent on its essential amino acid composition. The results of the quantitative determinations of the various amino acids of germinated and ungerminated fenugreek seeds are

shown in Table 1 which indicates the presence of the same amino acids in both ungerminated and germinated seeds. Leucine, lysine, arginine, proline, aspartic and glutamic acids were the richest amino acids in fenugreek. The high percentages of glutamic and aspartic acids are normal for most seeds and this correlated with the results previously reported by Sauvaire *et al.* (1976) for fenugreek seeds. It is evident from Table 1 that, after germination, four of the essential amino acids decreased while phenylalanine, tyrosine, threonine, tryptophan and valine increased. Aspartic acid showed a greater increase, amounting to 83% of the

**TABLE 1**
Total Amino Acid Contents of Fenugreek Seeds as Affected by Germination

| *Amino acid* | *UGS* | | *GS* | |
|---|---|---|---|---|
| | *g/100 g Sample*[a] | *g/100 g Protein* | *g/100 g Sample*[a] | *g/100 g Protein* |
| Isoleucine | 0·834 | 3·40 | 0·352 | 1·19 |
| Leucine | 1·73 | 5·22 | 1·62 | 5·46 |
| Lysine | 1·07 | 4·36 | 1·24 | 4·17 |
| Methionine | 0·204 | 0·833 | 0·203 | 0·685 |
| Cystine | 0·526 | 2·15 | 0·389 | 1·31 |
| Phenylalanine | 0·767 | 3·13 | 1·14 | 3·85 |
| Tyrosine | 0·494 | 2·02 | 0·634 | 2·14 |
| Threonine | 0·319 | 1·30 | 0·888 | 3·00 |
| Tryptophan | 0·288 | 1·18 | 0·369 | 1·25 |
| Valine | 0·695 | 2·84 | 0·968 | 3·27 |
| Arginine | 1·57 | 6·40 | 1·58 | 5·35 |
| Histidine | 0·409 | 1·67 | 0·643 | 2·17 |
| Alanine | 0·678 | 2·77 | 0·806 | 2·72 |
| Aspartic acid | 1·80 | 7·35 | 4·00 | 13·5 |
| Glutamic acid | 2·74 | 11·2 | 2·19 | 7·40 |
| Glycine | 0·388 | 1·58 | 0·778 | 2·63 |
| Proline | 1·01 | 4·12 | 0·389 | 1·31 |
| Serine | 0·877 | 1·54 | 0·835 | 2·82 |
| Ammonia | 0·052 | 0·212 | 0·855 | 2·89 |
| Total nitrogen (%) | 3·92 | | 4·74 | |
| Crude protein (N × 6·25) | 24·5 | | 29·6 | |

[a] On a dry weight basis where the moisture contents of ungerminated and germinated seeds were 9·66% and 5·87%, respectively.

original content. At the same time after germination the ammonia content increased fourteen times. The increasing percentage of aspartic acid in the germinated seeds may be derived from the hydrolysis of its amide (Chen & Thacker, 1978). On the other hand, glutamic acid and proline showed marked decreases after germination, amounting to 34% and 68%, respectively. These findings differ from those of Chen & Thacker (1978) who found that proline and tryptophan showed a marked decrease after germination of peas for 5 days.

The effects of germination on the individual free amino acid content of fenugreek seeds are shown in Table 2. Glycine, lysine and glutamic acid account for 50% of the free amino acids of the ungerminated seeds. All the free amino acids increased after germination for 120 h, while glycine decreased. On the other hand, cystine, tryptophan and proline originated

**TABLE 2**
Effect of Germination on the Individual Free Amino Acids of Fenugreek[a]

| *Amino acid* | *UGS* | | *GS* | |
|---|---|---|---|---|
| | *mg/100 g Sample* | *mg/100 g Protein* | *mg/100 g Sample* | *mg/100 g Protein* |
| Isoleucine | 4 | 17 | 49 | 165 |
| Leucine | 5 | 20 | 51 | 172 |
| Lysine | 67 | 275 | 73 | 246 |
| Methionine | 3 | 14 | 0 | 0 |
| Cystine | 0 | 0 | 24 | 81 |
| Phenylalanine | 19 | 77 | 143 | 483 |
| Tyrosine | 12 | 50 | 55 | 186 |
| Threonine | 10 | 42 | 79 | 266 |
| Tryptophan | 0 | 0 | 21 | 71 |
| Valine | 4 | 18 | 156 | 526 |
| Arginine | 40 | 161 | 60 | 202 |
| Histidine | 12 | 49 | 41 | 138 |
| Alanine | 17 | 71 | 171 | 577 |
| Aspartic acid | 14 | 56 | 99 | 335 |
| Glutamic acid | 30 | 122 | 56 | 189 |
| Glycine | 93 | 379 | 88 | 297 |
| Proline | 0 | 0 | 202 | 682 |
| Serine | 9 | 37 | 320 | 1 080 |
| Ammonia | 20 | 81 | 77 | 260 |

[a] Average of three determinations calculated on a dry weight basis.

in a free form after germination, as previously reported by El-Mahdy & El-Sebaiy (1982). This increase is mainly due to the proteolytic action of enzymes which break down the complex protein molecules into simpler units of amino acids. The greatest increases in the free amino acids were observed in the amounts of serine, alanine, phenylalanine, valine, aspartic acid and threonine, while other amino acids did not change significantly during germination. This may reflect a balance between synthetic and degradation processes. The simplest explanation is that they are liberated from reserve proteins and utilized more or less unchanged (Hegazi, 1974). The increase in the ammonia content of germinated seeds suggested that some of the liberated amino acids were oxidatively deaminated.

**Germination and protein quality**

The quality of a protein is determined not only by its amino acid pattern, but also by its digestibility, the relative proportions of different amino acids and their availability, the presence of indigestible carbohydrates and of anti-nutritional factors such as trypsin inhibitor (Venkataraman *et al.*, 1976).

Like most leguminous plants, fenugreek is expected to be deficient in the sulphur-containing amino acids and tryptophan (Jamalian & Pellett, 1968; Subramanian *et al.*, 1976). The data in Table 3 reveal that fenugreek seed proteins are deficient in threonine, as the first limiting amino acid, and in valine, as the second. After germination the picture changes, the first limiting amino acid becomes isoleucine and the next, the sulphur-containing amino acids. Tryptophan remains unchanged, while threonine approximately doubles. If one considers the total amounts of the sulphur-containing amino acids, the A/E ratios are 114 for ungerminated, 76 for germinated and 98 for the FAO provisional pattern (FAO, 1973).

The chemical score—based on the essential amino acid pattern of whole egg protein—for fenugreek seeds showed a marked decrease (21 %) after germination.

*In vitro* pepsin, followed by pancreatin digestion, gives a realistic picture of protein digestibility in the gastrointestinal tract. As shown in Table 4, the *in vitro* digestibility of germinated seed proteins showed a slight increase over the ungerminated.

The C-PER (computed PER) assay, which utilizes the essential amino acid profile and *in vitro* protein digestibility data to predict the protein quality, was found to be 0·36 for ungerminated fenugreek seeds.

**TABLE 3**

Essential Amino Acid Patterns, A/E Ratios and Amino Acid Scores of Fenugreek Seeds Before and After Germination

| *Essential amino acid* | *Milligrams of amino acid per gram of nitrogen* | | | | | *A/E ratio*[b] | | | | *Amino acid scores* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *FAO*[a] *pattern (1973)* | *Whole*[a] *egg* | *Casein* | *UGS* | *GS* | *FAO pattern* | *Casein* | *UGS* | *GS* | *UGS* | *GS* |
| Isoleucine | 250 | 340 | 350 | 213 | 74 | 111 | 109 | 129 | 45 | 60·8 | 21·1 |
| Leucine | 440 | 540 | 568 | 326 | 341 | 195 | 178 | 197 | 207 | 57·4 | 60·0 |
| Lysine | 340 | 440 | 506 | 272 | 261 | 151 | 158 | 165 | 159 | 53·8 | 51·6 |
| Methionine + Cystine | 220 | 355 | 300 | 188 | 125 | 98 | 94 | 114 | 76 | 62·7 | 41·7 |
| Phenylalanine + Tyrosine | 380 | 580 | 678 | 322 | 374 | 169 | 215 | 195 | 227 | 47·5 | 55·2 |
| Threonine | 250 | 294 | 275 | 81 | 187 | 111 | 86 | 49 | 114 | 29·5 | 68·0 |
| Tryptophan | 60 | 106 | 79 | 74 | 78 | 27 | 24 | 45 | 47 | 93·7 | 98·0 |
| Valine | 310 | 410 | 425 | 177 | 204 | 138 | 133 | 107 | 124 | 41·6 | 48·0 |
| Total essential amino acids | 2250 | 3065 | 3190 | 1653 | 1644 | | | | | | |
| Amino acid score[c] | | | | 29·5 | 21·1 | | | | | | |
| Chemical score[d] | | | | 27·6 | 21·8 | | | | | | |

[a] Pellet & Young (1980).

[b] A/F ratio; the relationship between the content of an individual amino acid in food protein (A) and the total essential amino acid content (E).

[c] $\text{Amino acid score} = \dfrac{\text{Milligrams of essential amino acid per gram of test protein}}{\text{Milligrams of amino acid in one gram of reference protein (casein)}} \times 100$

[d] $\text{Chemical score} = \dfrac{\text{Milligrams of essential amino acid per gram of test protein}}{\text{Milligrams of essential amino acid per gram of whole egg protein}} \times 100$

**TABLE 4**
*In Vitro* Digestibility and PER Values of Fenugreek Seed Proteins and Casein as Affected by Germination

| | *Digestibility*[a,b] | *C-PER absolute* |
|---|---|---|
| Ungerminated fenugreek | 74·1 | 0·36 |
| Germinated fenugreek | 76·4 | 0·34 |
| Casein | 80·8 | 2·51 |

[a] Pepsin followed by pancreatin.
[b] Average of three determinations.

Germination of fenugreek seeds lowered the C-PER, a result which indicates that the protein quality decreased.

In conclusion, the present study showed that, after 120 hours' germination of fenugreek seeds, the free amino acids, proteolytic activity, anti-tryptic activity and protein digestibility increased. No improvement in the protein quality could be detected, *in vitro*, as a result of germination.

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