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Protein Concentrates from Italian Millet (*Setaria italica*) and Their Enzymatic Hydrolysis

D. Hara Gopal

Division of Biological Sciences, University of Missouri, Columbia, Missouri, USA

P. Vincent Monteiro, T. K. Virupaksha* & Geeta Ramachandra

Department of Biochemistry, University of Agricultural Sciences, GKVK Campus, Bangalore-560 065, India

ABSTRACT

Protein concentrates were prepared from three varieties of Italian millet by extraction of lipid-free whole millet flour with 70% isopropanol with a recovery of 11%–25% of the protein. The protein concentrates contained large amounts of non-essential amino acids. The amounts of essential amino acids in the concentrates were substantially higher than in wholeseed protein except for lysine and arginine. Amino acid scores of the protein concentrate from the variety CO-3 were superior to those from the varieties K-221-1 and I.Se-201. Protein concentrates had much lower in vitro *protein digestibilities (IVPD) with trypsin than with pepsin and papain.*

INTRODUCTION

Italian millet (*Setaria italica*) is grown extensively in India where it is used for both food and feed, in contrast to North America where it is grown mostly as a forage crop. Italian millet has a high fibre and lipid content (Monteiro *et al.*, 1988) when compared to other minor millets, which may result in rapid rancidification of the milled whole flour. The keeping quality of the milled flour is limited.

Due to economic reasons, plant foodstuffs play a major role in supplying

* To whom all correspondence should be addressed.

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the protein needs of the fast-increasing world population. Knowledge of the nutritional quality of these plant proteins must come from biochemical studies, so that they may be used either as a major source of protein or as a supplement with other proteins. Protein concentrates are finding increased use in processed foods and have been prepared from wheat (Wu & Sexson, 1975), rice (Connor *et al.*, 1976), sorghum (Wu, 1978) and pearl millet (Bailey & Sumrell, 1980). Digestibility of protein concentrates with proteolytic enzymes is also a factor which needs to be explored since nutritive value of the protein or protein utilisation depends on the ability of a protein to provide amino acids at levels similar to those of body proteins.

This paper describes the preparation of protein concentrates from Italian millet, their amino acid composition, studies on the *in vitro* protein digestibility and the time course of hydrolysis of the protein concentrates.

MATERIAL AND METHODS

Samples

Three varieties of Italian millet, K-221-1, I.Se-201 and CO-3, grown in the same season and location under standard agronomic practices, were obtained from the Plant Scientists (Millets), University of Agricultural Sciences, Bangalore, India. The varieties were chosen randomly from the germplasm collection. These varieties are considered suitable for cultivation under various agroclimatic conditions and are high-yielding varieties. The seeds were dehulled by abrasion in a McGill Sheller and milled to pass through a 60-mesh sieve. The flour was homogenised in petroleum ether (40°–60°C) and the lipids removed by decanting the supernatant. The defatted flour was air-dried.

Enzymes

Pepsin (1:3000, B.P.) was purchased from SD's Fine Chemicals, India, trypsin was a product of E. Merck (West Germany) and papain was from Sigma Chemical Co., USA.

Isolation of protein concentrates

Protein concentrates were prepared from defatted flour of the three Italian millet varieties by the procedure of Bailey & Sumrell (1980). Forty grams of defatted flour was extracted overnight with 400 ml of 70% (v/v) aqueous isopropanol (IPrOH) at room temperature. After centrifugation to separate the supernatant, the residue was washed twice with 200 ml portions of 70%

IPrOH and the supernatants were combined and dialysed extensively against distilled water at room temperature until free from IPrOH. The dialysate was centrifuged to collect the precipitated protein which was then suspended in a small volume of water and lyophilised to give the protein concentrate. Nitrogen in the concentrates was determined by the micro-Kjeldahl method (Bailey, 1972) and protein content calculated (% N × 6·25).

Amino acid analyses

Protein concentrates were hydrolysed in evacuated sealed tubes in 6N HCl at 110°C for 22 h and amino acids were determined on a Hitachi Amino Acid Analyser, Model KLA-3B, according to Spackman *et al.* (1958). Tryptophan was not estimated.

IVPD of protein concentrates

Pepsin, trypsin and papain were used for IVPD studies.

IVPD with pepsin was determined by the AOAC (1980) procedure. Ten milligrams of protein concentrates were weighed into a series of test tubes and 1·0 ml of 0·075N HCl and 0·1 ml of pepsin solution (2·0 mg/ml) in 0·075N HCl were added to each tube. The tubes were incubated at 37°C and enzyme action was stopped at 0, 10, 30, 60 and 1440 min addition of 1 ml of 10% trichloroacetic acid (TCA). The reaction mixture was filtered through Whatman No. 54 filter paper, the nitrogen in the washed residue estimated by the micro-Kjeldahl procedure and IVPD was calculated.

Per cent hydrolysis with trypsin was determined essentially as described for pepsin, except that incubation was in 0·1M phosphate buffer, pH 7·6.

Digestibility of the protein concentrates with papain was determined by the method of Buchanan & Byers (1969) as modified by Nanda *et al.* (1977). Ten milligrams of protein concentrate were incubated with 0·4 ml papain solution (25 mg/ml), 0·5 ml thioglycollic acid reagent and 1·0 ml of phosphate–citrate buffer, pH 6·6. The rest of the procedure adopted was the same as with pepsin.

All digestions were performed in triplicate and suitable blanks were included.

Electrophoresis of enzyme digests of protein concentrates

Pepsin and trypsin were used to obtain enzyme digests for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrates were incubated with the appropriate enzyme, under the pH and temperature conditions described for determination of IVPD, at an enzyme

to concentrate ratio of 1:50. At 0, 10, 30, 60 and 300 min intervals, aliquots were withdrawn from the reaction mixtures into an equal volume of sample buffer for SDS-PAGE (Weber & Osborn, 1969) and placed in a boiling water bath for 5 min. Electrophoresis was carried out in 10% gels.

RESULTS AND DISCUSSION

Prolamin being the major storage protein of Italian millet (Monteiro *et al.*, 1982), 70% aqueous IPrOH was used for isolation of protein concentrates from whole flour. The protein contents of the concentrates are presented in Table 1 and compared with protein contents of whole flour (Monteiro *et al.*, 1982). Protein contents of the concentrates ranged from 48·4% to 76·9%

TABLE 1
Recovery of Protein in Concentrates from Italian Millet Cultivars

Cultivar	*Crude protein in whole seed*[a] (%)	*Crude protein in concentrate* (%)	*Protein recovery* (%)
I.Se-201	14·4	68·9	14·1
K-221-1	12·3	48·4	11·4
CO-3	14·3	76·9	25·6

[a] Values from literature (Monteiro *et al.*, 1982).

representing a recovery of only 11·4%–25·6% of total protein, though the prolamin content of whole flour of these Italian millet varieties ranged from 61% to 68% (Monteiro *et al.*, 1982). Quantitative recovery of the major storage protein (prolamin) of Italian millet requires successive extraction with several solvents. Such procedures are not suitable for the preparation of protein concentrates from whole flour since commercial exploitation requires a simple and rapid procedure. Extraction with 70% aqueous IPrOH leads to the recovery of the true-prolamin fraction and not the prolamin-like fraction. Recovery of the latter requires extraction with 70% aqueous IPrOH containing mercaptoethanol (Monteiro *et al.*, 1982).

Amino acid composition of protein concentrates

Amino acid composition of the three protein concentrates is summarised in Table 2. Amino acid composition of the whole flour of the three varieties is also included for comparison. Only minor differences exist in the amino acid

TABLE 2
Amino Acid Composition[a] of Wholeseed Protein[b] and Protein Concentrates of Italian Millet[c]

Amino acid	*I.Se-201*		*K-221-1*		*CO-3*	
	Whole seed	*Protein concentrate*	*Whole seed*	*Protein concentrate*	*Whole seed*	*Protein concentrate*
Lysine	2·4	Traces	3·1	Traces	2·3	Traces
Histidine	3·1	2·7	3·6	2·7	2·8	2·6
Arginine	5·6	3·6	5·9	3·8	5·8	2·5
Aspartic acid	7·3	8·0	7·9	6·7	7·2	8·7
Threonine	3·7	3·7	4·0	5·6	3·7	6·4
Serine	5·2	6·3	5·4	6·0	4·3	6·7
Glutamic acid	25·1	34·1	32·4	32·6	19·7	34·8
Proline	7·6	11·7	10·4	14·6	6·4	11·9
Glycine	2·2	2·1	3·4	2·3	2·1	1·6
Alanine	8·5	13·7	11·5	13·4	8·1	13·9
Half-cystine[d]	0·5	2·9	Traces	3·0	0·4	1·9
Valine	4·5	5·1	5·7	4·5	4·7	5·4
Methionine	1·9	7·9	2·7	6·7	2·1	4·2
Isoleucine	3·7	6·1	4·2	5·6	4·1	6·1
Leucine	15·3	21·3	18·4	19·9	14·2	28·7
Tyrosine	2·4	5·6	2·5	5·3	2·6	5·6
Phenylalanine	4·3	11·3	5·6	10·1	4·7	9·7

[a] Expressed as mg per 16 mg N.
[b] Values from literature (Monteiro *et al.*, 1982). These analyses were done on the same batch of millet samples which were also used for the preparation of protein concentrates.
[c] Recovery of amino N was in the range 84–89%.
[d] Half-cystine includes cysteic acid. Cystine in the samples was partly oxidised to cysteic acid during the preparation of hydrolysates and was eluted in the position of authentic cysteic acid.

make up of the concentrates. It is notable that all the protein concentrates are highly deficient in lysine and reflect the amino acid composition of the prolamin fraction of the millet. However, the histidine and arginine contents of these protein concentrates are higher than are usually found in prolamins of cereals and millets. As is usual for the prolamin fraction, the protein concentrates have high levels of glutamic and aspartic acids, proline, alanine and leucine.

The nutritional quality of the Italian millet protein concentrates was evaluated by the FAO scoring pattern (FAO, 1973) and is shown in Table 3. The essential amino acid scores of the concentrates are, for the most part, better than that of whole flour, except for valine and lysine. Although the concentrates contain large amounts of most of the essential amino acids,

TABLE 3
Essential Amino Acid Scores of Italian Millet Protein Concentrates

Amino acid	*Amino acid scores for protein concentrates*			*Italian millet whole seed flour*[a]	*Provisional FAO scoring pattern*[b]
	I.Se-201	*K-221-1*	*CO-3*		
Isoleucine	153	140	152	104	40
Leucine	305	284	409	236	70
Lysine	—	—	—	47	55
Methionine + Cystine	143	191	120	70	35
Phenylalanine + Tyrosine	185	169	161	125	60
Threonine	91	139	161	94	40
Valine	100	90	108	108	50

[a] Values from literature (Monteiro *et al.*, 1982). See footnote *b* (Table 2).
[b] From FAO Nutrition Meetings Report No. 52 (1973).

$$\text{Amino acid score} = \frac{(\text{mg of amino acid in 1 g of protein})}{(\text{mg of amino acid suggested})} \times 100$$

the severe deficiency of lysine makes them nutritionally unsuitable for use as the major or sole source of protein.

Comparing the amino acid compositions of the protein concentrates with total protein of the respective varieties, it can be seen that the non-essential amino acids such as glutamic acid, proline and alanine are present in larger amounts in the concentrates but the levels of essential amino acids, lysine and arginine, are considerably less. However, methionine, isoleucine, leucine, tyrosine and phenylalanine levels of the concentrates are much higher than in whole flour protein.

Amino acid composition of proteins plays an important role in determining the nutritive value of protein foodstuffs. An excess or deficiency of certain amino acids can be nutritionally deleterious (Harper & Benevenga, 1970). This may have some practical significance. It has been shown that excess leucine interferes with the utilisation of isoleucine (Harper, 1955). In the three protein concentrates the leucine to isoleucine ratios are 3·48, 3·55 and 4·71 for the varieties I.Se.-201, K-221-1 and CO-3, respectively, and the relatively high amounts of leucine may result in an antagonism in the utilisation of isoleucine. Arginine levels in Italian millet protein concentrates are much higher than lysine and may result in poor utilisation of lysine (Ganapathy & Chitre, 1970).

In vitro protein digestibility

Protein digestibility is essentially a measure of the rate of protein hydrolysis by digestive enzymes. Protein digestibility is a factor most likely to affect amino acid availability. Proteolysis is influenced both by the linear amino acid sequence and the tertiary structure of a protein.

Per cent hydrolyses of the protein concentrates with pepsin, trypsin and papain are shown in Table 4. With pepsin, as much as 11% hydrolysis was observed at zero time. This could be due to a time lag between addition of

TABLE 4
Per cent Hydrolysis of Protein Concentrates with Pepsin, Trypsin and Papain

Time	*I.Se-201*			*K-221-1*			*CO-3*		
	Pepsin	*Trypsin*	*Papain*	*Pepsin*	*Trypsin*	*Papain*	*Pepsin*	*Trypsin*	*Papain*
0 min	11·0	9·8	9·9	6·9	6·9	6·3	4·3	8·0	5·3
10 min	30·5	16·3	28·3	35·9	10·1	24·4	8·3	12·6	26·6
30 min	34·8	20·0	35·8	59·4	12·9	35·9	31·8	14·1	30·8
60 min	54·7	20·2	54·7	61·8	17·6	59·4	41·7	16·3	50·6
24 h	60·4	23·3	60·4	69·0	22·8	61·8	67·5	20·2	59·3

enzyme to the reaction mixture and addition of TCA for inactivation. It may also be possible that the protein concentrates contain low molecular weight components not precipitated by TCA, resulting in the high values observed for hydrolysis at zero time. After 60 minutes' incubation the extent of hydrolysis was 42% to 62% for the three concentrates. Further incubation for a total of 1440 min (24 h) resulted in only a marginal increase in hydrolysis over the 60 min digestion.

In the case of trypsin also, most of the digestion takes place in 60 min and further incubation up to 24 h results only in slightly increased hydrolysis.

Digestibility of the concentrates with papain follows the same pattern as with pepsin. Approximately 60% of the protein was hydrolysed in 24 h which does not represent much of an increase over a 60-min hydrolysis.

Digestibilities of the concentrates with pepsin and papain range from 60% to 69%, while with trypsin they are much lower, being only 20% to 23%. It is well known that trypsin exhibits a strict specificity for lysyl —*X* and arginyl —*X* peptide bonds and, therefore, low levels of these amino acids must necessarily be reflected in a low trypsin digestibility. Further, lysylprolyl and arginylprolyl bonds are completely resistant to trypsin (Bell, 1954). It is thus quite conceivable that the high proline levels of the concentrates, coupled with the low levels of lysine and arginine, may favour

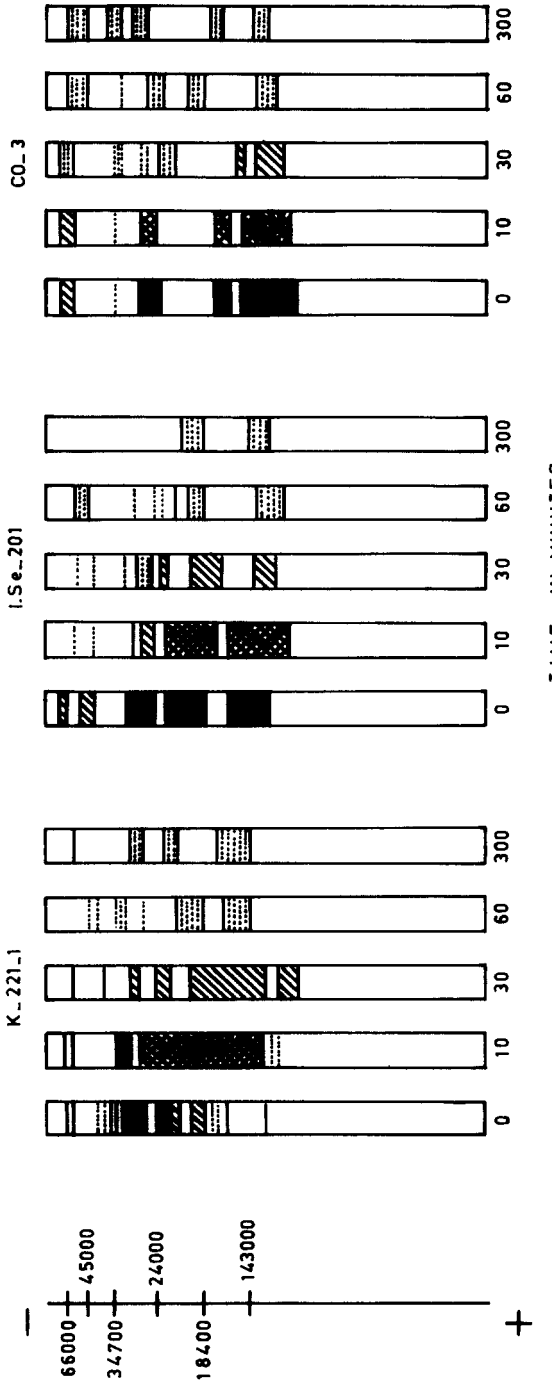

Fig. 1. Time course of hydrolysis of protein concentrates of Italian millet by pepsin, monitored by SDS-PAGE. Migration is from top (cathode) to bottom (anode). Solid bars represent the most intense protein bands, cross-hatched bars moderately intense and stippled bands the least intense protein bands.

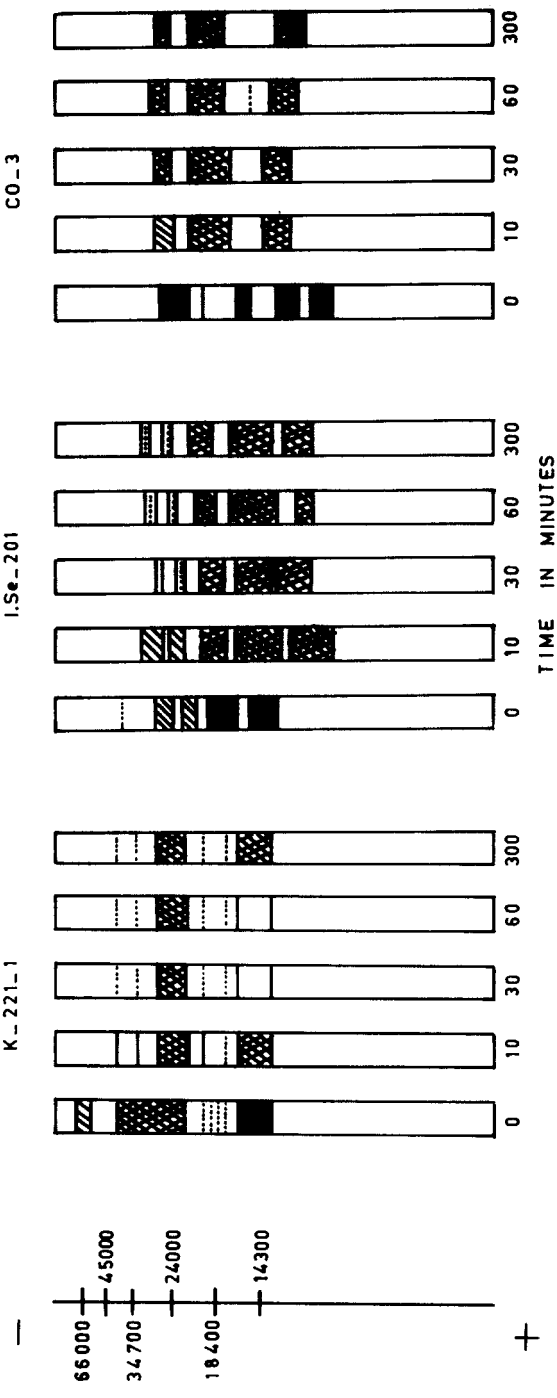

Fig. 2. Time course of hydrolysis of protein concentrates of Italian millet by trypsin, monitored by SDS-PAGE. Migration is from top (cathode) to bottom (anode). Solid bars represent the most intense protein bands, cross-hatched bars moderately intense and stippled bands the least intense protein bands.

a preponderance of such linkages in the millet protein concentrates, resulting in low IVPD values with trypsin. In all cases, enzymic digestion for 60 min resulted in almost complete digestion and extending the incubation period to 24 h did not appreciably increase digestibility of the concentrates. From this it may be concluded that 60 minutes' digestion is sufficient to judge the nutritional quality of a protein by IVPD studies. The method of Axtell *et al.* (1981) for determination of digestibility of sorghum proteins with pepsin also lends support to this view.

Time course of hydrolysis of protein concentrates

SDS-PAGE patterns of pepsin and trypsin digests of the protein concentrates are shown in Figs 1 and 2, respectively. From the zero minute pattern it is clear that the concentrates contain a heterogeneous mixture of polypeptides in the molecular weight (MW) range 14 000–66 000. In the pepsin digests, with increase in digestion time, polypeptides of smaller MW increased in number. The presence of bands in the 300 min sample showed that some protein remained undigested even after prolonged incubation with pepsin. However, the absence of low molecular weight subunits in the PAGE-patterns expected to be formed on progressive degradation of the protein may indicate that the hydrolysis products of pepsin digestion were small enough to diffuse out of the gel.

Gel patterns of tryptic digests did not show much variation from the zero time sample to the 300 min sample, in contrast to the SDS-PAGE patterns of the pepsin digests. This could be due to the low susceptibility of the proteins in the concentrates to tryptic digestion. This further confirms the results of IVPD studies in which IVPD with trypsin was found to be low. Lynch *et al.* (1977) have also shown that glycinin of soybean is at least tenfold less susceptible to trypsin than pepsin.

CONCLUSION

The results of these studies show that, though Italian millet contains high amounts of prolamin, only 11%–25% of the protein could be recovered by aqueous isopropanol extraction. The protein concentrates contain large amounts of most of the essential amino acids but the overall amino acid composition is not well balanced from the nutritional point of view due to the severe deficiency of lysine. IVPD with pepsin and papain is almost complete but IVPD with trypsin is low. However, the high digestibility with pepsin may not be very significant physiologically since peptic digestion of

food occurs for a very short length of time. Again, the high digestibility of the concentrates with papain is not relevant in human or animal nutrition since papain is not a digestive enzyme in these species.

The low digestibility of the concentrates with trypsin makes the concentrates unsuitable for inclusion in the diet without some preliminary processing to make the amino acids more easily available.

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