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Examination and Modification of the Use of *Leuconostoc mesenteroides* for Measurements of the Sulfur-Containing Amino Acids from *Vigna unguiculata*

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The use of *Leuconostoc mesenteroides* for the measurement of methionine and cyst(e)ine from the seed proteins of the legume *Vigna unguiculata* as well as *Phaseolus vulgaris* was examined and modified. Principal modifications include the use of buffers in neutralization and Pronase hydrolysis. Pronase appears useful in the hydrolysis of seed proteins of *V. unguiculata* but not for those from *P. vulgaris*. The method, as modified here, recovers as much methionine as that measured by an amino acid analyzer. It was found that hydrolysis preceding amino acid measurement need not be complete since the bacteria can obtain methionine from di- and tripeptides. Another advantage of this assay is that methionine derivatives, known to be nutritionally less desirable when fed to chicks, are shown to be less desirable when fed to this bacterium. Bacterial growth rates obtained on each of the derivatives mimic those reported for chicks. Because of differential growth given by equal amounts of cysteine and cystine, this method cannot give reliable measurements of these two amino acids. Further studies of this anomalous result suggest that cysteine must be converted to cystine before it supports bacterial growth. Other problems in the use of this assay are discussed.

When monogastric animals digest proteins from legumes, methionine appears to be the first limiting essential amino acid (Boulter et al., 1973). Cystine (cysteine) can apparently partially alleviate the requirement for methionine. Thus, any effort to improve the protein quality of legumes by genetic means must concentrate on the sulfur amino acids. Technically, a search for rare genotypes with elevated levels of these amino acids requires a rapid, reliable method for their measurement. The amino acid analyzer is not ideal since analysis is time consuming, exhaustive hydrolysis sometimes destroys methionine, and methionine derivatives known to be nutritionally less desirable in comparison to methionine are measured as methionine. An alternative method involves use of the bacterium *Leuconostoc mesenteroides*. This bacterium in a microbiological assay appears promising because a strain is available that requires both methionine and cystine (cysteine), and the appropriate growth media are available commercially. Moreover, this bacterium has been used for the measurement of these amino acids (Steel et al., 1949; Evans et al., 1974).

Although this bacterium has been used to measure the S-amino acids, we have found no literature pertaining to the accuracy and sensitivity of this method in measuring the total S-amino acid content. Thus, we have examined this microbiological assay, as well as hydrolysis preceding measurement, for the analysis of methionine and cystine from the legume, *Vigna unguiculata* (cowpea). Procedural modifications have been introduced to reduce the time required for amino acid measurement. The results of these studies, which are the subject of this report, strongly suggest that this method should be useful in programs where many samples are to be analyzed for methionine content.

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MATERIALS AND METHODS

The bacterium *Leuconostoc mesenteroides* ATCC 8042 was obtained from Difco Lab., Detroit, Mich., as were the methionine (Difco 423) and cysteine (Difco 467) growth media. Bacteria were routinely maintained in a 1:1 mixture of these media and in stab cultures of AOAC lactobacilli agar at 0–5 °C.

Materials for analysis were ground in a Udy mill, dried at 50 °C, and then stored in a desiccator. Hydrolysis of proteins was performed enzymatically or by means of 6 N HCl as described below.

Five milliliters of 6 N HCl was added to 100 mg of sample and the vessel was covered with a hard plastic Teflon-lined screw cap. Hydrolysis was carried out in an autoclave at 121 °C for 60 min. The samples were cooled, adjusted to pH 6.2 to 6.6 with 6 N NaOH, filtered with suction, and made up to volume (30–50 mL) with 0.2 M potassium–sodium phosphate (pH 6.4). The phosphate buffer does not affect bacterial growth.

For enzymatic hydrolysis, the enzyme solution was 0.01 M Tris-HCl, 0.15% Pronase (P-5130, Sigma), and 0.01 M $CaCl_2$ (pH 8.0). Five milliliters was added to 100 mg of sample and incubated at 37 °C for 1.5 h in a stationary water bath (continuous shaking does not increase the amount of amino acid recovery). After hydrolysis, the

Table I. Effect of Hydrolysis Atmosphere, and Storage before and after Neutralization, on Methionine and Cystine Amounts of Enriched Cowpea Meal

Entry	Amino acid added	Hydrolysis atm	Storage[a]	mg of Met/g	mg of Cys/g
1	Met (2.4 mg/g)	N_2	None	5.42 ± 0.09	1.27 ± 0.06
2	Met	N_2	N	5.71 ± 0.17	0.89 ± 0.05
3	Met	Air	None	5.29 ± 0.17	1.26 ± 0.05
4	Met	Air	N	5.15 ± 0.06	0.94 ± 0.04
5	Met	N_2	A	3.55 ± 0.16	0.62 ± 0.03
6	Cys (1 mg/g)	N_2	None	2.38 ± 0.03	2.91 ± 0.07
7	Cys	N_2	N	1.99 ± 0.08	2.25 ± 0.06
8	Cys	Air	None	2.38 ± 0.01	2.79 ± 0.05
9	Cys	Air	N	2.02 ± 0.07	2.28 ± 0.02
10	Cys	N_2	A	1.51 ± 0.02	1.98 ± 0.08

[a] Samples were either assayed immediately or stored 48 h in neutral (N) or acidic (A) solutions.

samples were filtered and made to volume (30–50 mL) with 0.12 M Tris-HCl (pH 6.3). The Tris-HCl buffers do not affect bacterial growth. Other enzymes used were Sigma products: trypsin, T-8253; pepsin, P-7000; papain, P-3375.

The methionine and cystine growth media were prepared as described by Difco. Standard growth tubes, which contained 0–10 μg of cystine (Sigma) or 0–24 μg of methionine (Sigma) per tube, were prepared and run in each experiment. When samples were prepared as described above, 0.5-mL aliquots for cystine and 1-mL aliquots for methionine were used routinely. After amino acid addition, each tube was fitted with an 18-mm plastic cap (Scientific Products, T-1396-3) which contained a 1-mm hole. The tubes were covered with aluminum foil and sterilized at 10 lb pressure for 4 min. After cooling, the tubes were inoculated with vigorously growing bacteria that had been previously washed twice by centrifugation in 0.85% NaCl.

After inoculation, tubes were stored at 37 °C for 40–63 h and absorbance at 700 nm was determined.

Chemical estimation of methionine was by means of ion-exchange chromatography. Samples were hydrolyzed in a sealed evacuated tube (0.05 Torr) in 6 N HCl at 105 °C for 22 h and analyzed on a Locarte autoanalyzer, as described previously (Evans and Boulter, 1974).

RESULTS AND DISCUSSION

Possible causes of amino acid loss during analysis were examined. The effects of hydrolysis atmosphere and storage times on recovery of methionine and cystine are shown in Table I. A comparison of samples hydrolyzed under a nitrogen atmosphere with those that were not shows little to no effect of this treatment on methionine or cystine. Hydrolysis without nitrogen flushing led to 96% of the methionine obtained with nitrogen. Flushing with nitrogen does not lead to increased recovery of cystine. A similar analysis shows that 99% of the cystine obtained from vessels flushed with nitrogen is recovered in the absence of this treatment.

The effect of storage was also determined. Five samples were hydrolyzed, neutralized, and immediately added to assay tubes. The assayed amounts of the amino acids are presented in entries 1, 3, 6, and 8 in Table I. The hydrolysates were then stored for 48 h; aliquots were taken and examined for their amino acid contents. These are shown in entries 2, 4, 7, and 9. Overall, the data show that storage in neutral solution leads to a 4% reduction in methionine whereas a 23% decrease in cystine content is observed.

As might be expected, storage of the hydrolysate in 6 N HCl for 48 h leads to substantial loss in amino acid content. The data show that this exposure leads to a 35% reduction in methionine content and 37% loss in cystine. In view of these storage data, it is recommended that sample hydrolysis and commencement of bacterial growth be done on the same day.

The possibility of amino acid loss during hydrolysis was also examined. In these experiments, known amounts of methionine or cystine were added to cowpea meal and the observed increase in amino acid content was compared to the expected. If methionine or cystine is lost during hydrolysis, the amount of the amino acid measured after hydrolysis should be less than the expected value. A comparison of samples with and without exogenous methionine (Table I) provides no evidence for methionine loss. Methionine levels in samples containing 2.4 mg of additional methionine were equal to 113% of the expected amount. Similarly, analysis of samples with and without 1.0 mg of exogenous cystine revealed that the measured level of cystine in the mixture was 122% of the expected value.

In other experiments (C. Hannah, unpublished results), 6 hydrolysates containing 2.4 mg of exogenous methionine plus cowpea meal yielded 4.49 mg of methionine whereas 2.19 mg was observed in hydrolysates not containing the extra methionine; the enriched hydrolysates contained 98% of the expected amount. Also, in this former study, hydrolysates enriched with cystine before hydrolysis yielded 101% of the expected value.

In conclusion, it appears that under the conditions employed here (hydrolysis time of 60 min) free methionine and cystine are not lost during hydrolysis even in the presence of oxygen. However, it should be emphasized that longer hydrolysis periods can cause loss of these amino acids. We have observed, using purified lysozyme, that increasing the hydrolysis time from 1 to 3 h leads to a reduction of 28 and 39% for methionine and cystine, respectively, in hydrolysis vessels that were flushed with nitrogen.

For Pronase hydrolysis, initial experiments (results not shown) showed that a time of 1.5 h at a concentration of 0.15% Pronase gave best results.

A comparison of the two methods of hydrolysis on subsequent methionine recovery is shown in Table II (columns 1 and 2). A similar comparison of cystine recovery was not made. Cowpea lines, unselected for the purpose of this comparison, were grown at the International Institute of Tropical Agriculture, Ibadan, Nigeria, and sample grinding and nitrogen determinations were carried out at the University of Durham. Among all samples, acid hydrolysis led to 1.315 g of methionine, whereas enzymatic hydrolysates averaged 1.359 g of methionine, or 103% of the value obtained by acid hydrolysis. A comparison of data obtained with the microbiological assay with those obtained by an amino acid analyzer is also shown. Among all samples, the microbiological assay, after enzymatic hydrolysis, yielded 98.6% of the methionine obtained by the amino acid analyzer. Although variation

Table II. Comparison of Methionine Values Obtained after Acid or Enzymatic Hydrolysis and Subsequent Microbiological Determination and by Amino Acid Autoanalyzer

Cowpea	Met, g/16 g of nitrogen		
	Acid hydrolysis	Enzym. hydrolysis	Autoanalyzer
IVu 1354	1.38	1.42	1.45
176	1.20	1.26	1.18
57	1.01	1.12	1.24
530	1.61	1.58	1.48
1423	1.31	1.49	1.39
95	1.21	1.38	1.36
Prima	1.32	1.39	1.42
30	1.31	1.34	1.56
37	1.19	1.20	1.23
51	1.57	1.42	1.43
2093	1.41	1.49	1.35
3629	1.24	1.36	1.40
1552	1.34	1.22	1.43
Av	1.315	1.359	1.379

between lines is small, both methods, in general, identify the same lines as being high or low.

As shown above, hydrolysis with acid or the Pronase preparation yields approximately equal amounts of methionine from these examined cowpea lines. Screening of approximately 200 other lines (C. Hannah, unpublished results) verifies this observation although two lines were found which reproducibly gave less methionine when hydrolyzed with Pronase. However, examination of nine unselected lines of *Phaseolus vulgaris* (common bean) showed that acid hydrolysis led to a recovery of 2.87 mg of methionine/g whereas Pronase yielded only 2.23 mg of methionine/g. Furthermore, for each sample the acid-derived methionine was greater than that from Pronase. This suggests that seeds of *Phaseolus vulgaris* contain an inhibitor of Pronase activity or that the proteins are resistant to Pronase hydrolysis. Using the most Pronase-resistant lines, attempts were made to overcome this inhibition by use of other proteolytic enzymes, either each alone or in combination with Pronase. These attempts proved unsuccessful. For example, one *Phaseolus* line yielded 2.48 mg of methionine/g by acid hydrolysis but only 1.06 after Pronase hydrolysis. Treatment with papain alone, under conditions in which an increase in papain concentration led to no additional increase in methionine from the sample, gave only 1.27 mg of methionine. If papain treatment was followed by the usual Pronase hydrolysis, only 1.40 mg of methionine was recovered from the sample. Using the same protocol, it was found that treatment with trypsin or pepsin, each by itself or followed by Pronase, yielded no more methionine than did acid hydrolysis. Furthermore, a mixture of only trypsin and Pronase yielded more methionine (5.43 mg) than the sum of the two enzymes incubated separately (0.3 mg + 1.78 mg). Apparently, trypsin is more prone to hydrolysis by Pronase than it is to trypsin and/or vice versa. Similar analyses show that this is also true with mixtures of pepsin and Pronase.

Since very mild hydrolysis (acid or Pronase) preceding bacterial assay gives rise to almost complete recovery of methionine from cowpea protein (Table II), the possibility that *Leuconostoc mesenteroides* possess some proteolytic activity was examined. Dialyzed bovine serum albumin (BSA) was either hydrolyzed with Pronase as described above or treated with the Tris-HCl buffer which contained $CaCl_2$ (no Pronase). From 50 mg of BSA, 288 μg of methionine and 1006 μg of cystine were measured following Pronase hydrolysis. Omission of Pronase hydrolysis led to the recovery of 12 μg of methionine and 5 μg of cystine. Thus, *Leuconostoc mesenteroides* has little to no proteolytic activity.

Table III. Methionine Content of Various Peptides, as Measured by Growth of *L. mesenteroides*[a]

Compound	Rep. 1	Rep. 2
L-Methionyl-L-alanyl-L-serine	86.3	78.3
L-Methionyl-L-glutamic acid	79.0	85.8
L-Methionyl-L-asparagine	85.7	77.7
L-Methionylglycine	108	104
L-Methionyl-L-leucine	114	104
L-Methionyl-L-alanine	113	105
L-Methionyl-L-methionine	110	91.5

[a] Data are expressed as a percent of that expected if all the methionine from the peptide supports bacterial growth.

Although *L. mesenteroides* lacks the ability to hydrolyze large proteins, it apparently has the ability to obtain methionine from di- and tripeptides. Six dipeptides and one tripeptide were analyzed for their methionine content by use of *L. mesenteroides* without prehydrolysis. As seen in Table III, all peptides supported bacterial growth almost to the level expected if all the methionine were taken from the peptides. It would appear then that complete protein hydrolysis by acid or Pronase treatment is unnecessary since *L. mesenteroides* can obtain methionine from small peptides. The size of peptide required to prevent methionine utilization is presently unknown.

The mechanism by which *L. mesenteroides* utilizes methionine from small peptides is presently unknown. However, with other peptides it is known, by use of nongrowing cells and differentially labeled amino acids and peptides, that the peptides are taken up by the bacteria and then are cleaved within the cells (see, for example, Mayshak et al., 1966).

For nutritional studies, another advantage of the use of *Leuconostoc mesenteroides* in measuring methionine is pointed out below. Walker et al. (1975) recently discussed the fact that there exist, in biological materials, derivatives of methionine which have less nutritional value in comparison to methionine. Furthermore, some of these derivatives are converted to methionine or methionine sulfone during hydrolysis and thus are measured as methionine by the amino acid analyzer. Several methionine derivatives were supplied to *Leuconostoc mesenteroides* and it was observed that methionine sulfone completely lacks the ability to substitute for methionine while L-methionine sulfoxide and DL-methionine sulfoxide substitute to the extent of 85 and 60%, respectively. Almost identical results were obtained by Walker et al. (1975) when these derivatives were fed to chicks. Methionine sulfone was unable to satisfy the methionine requirement whereas L-methionine sulfoxide and DL-methionine sulfoxide substituted to the extent of 77 and 59%, respectively.

To determine whether the growth requirement for cystine could be satisfied by cysteine and, if so, whether quantitative growth response was identical for both amino acids, growth was measured at different levels of cysteine and cystine. Two lots of each amino acid were used and these were found to give identical results. Correction was made for the fact that cysteine was obtained as cysteine·HCl. As can be seen in Table IV, the cystine requirement can, as expected, be met by cysteine. However, more growth per microgram of amino acid was obtained with cystine. This is in contrast to the work of Risen et al. (1947), who reported equal growth of *L. mesenteroides* from cysteine and cystine. The results reported here might seem unexpected since the reduced form, cysteine, is

Table IV. Comparison of Growth of *Leuconostoc mesenteroides* Obtained with Different Amounts of Cysteine and Cystine

μg of cysteine	μg of cystine required to give growth equal to the corresponding amount of cysteine	No. comparisons	%
1	0.83	6	83
2	1.44	5	72
3	2.44	6	81
4	2.87	4	72
5	4.13	5	83
6	5.17	4	86
8	7.66	4	96
10	10.00	4	100

Table V. Effect of 2-Mercaptoethanol on Methionine-, Cystine-, and Cysteine-Dependent Bacterial Growth

Amino acid (μg)	2-Mercaptoethanol, M	Rel bacter. growth
Methionine (8)	0	28
Methionine (8)	0.0128	28
Methionine (8)	0.064	27
Methionine (8)	0.128	13
Methionine (8)	0.256	0
Cystine (4)	0	46
Cystine (4)	0.0128	1
Cystine (4)	0.064	1
Cystine (4)	0.128	1
Cysteine (3.2)	0	17
Cysteine (3.2)	0.0128	0
Cysteine (3.2)	0.064	0
Cysteine (3.2)	0.128	0

ultimately used in protein synthesis. Thus, cystine must be reduced before it is available for protein manufacture.

To investigate this further, the effects of 2-mercaptoethanol and dithiothreitol, reducing agents, on cystine- and cysteine-dependent growth were studied. As shown in Table V, 2-mercaptoethanol between 0.0128 and 0.128 M completely inhibited growth when cysteine or cystine was the limiting amino acid. When methionine was limiting, however, much higher concentrations of 2-mercaptoethanol (0.256 M) were required for complete inhibition of growth. Similarly, dithiothreitol at 10^{-3} to 10^{-2} M completely inhibited growth when cystine was present in low levels but had no effect when cystine was not the limiting amino acid. Since the two growth media are identical except for the presence of 500 μg of cystine/tube in the methionine medium and 1 mg of methionine/tube in the cystine medium, the differential effect of the reducing agents on bacterial growth must be related to the different levels of methionine and cystine in the two media. Thus, the data suggest that at a low level, 2-mercaptoethanol and dithiothreitol inhibit growth by specifically interfering with cystine and cysteine metabolism or uptake since its effect is seen only when these amino acids are limiting. The simplest explanation is that the reducing agents inhibit cystine formation and that cysteine must be oxidized to cystine before it supports bacterial growth.

One explanation is that cystine is the form needed for transport into the bacterial cell. Thompson and Meister (1975) have suggested, from kinetic characterization of an enzyme thought to be important in cystine breakdown and from levels of cysteine and cystine inside and outside mammalian cells, that cystine at low concentrations would be the preferred form of transport.

Since cystine supports more growth than cysteine, the relative amounts of both forms must be known before this microbiological system can be used to quantify levels of these amino acids. If the conversion of cysteine to cystine is the limiting step, as the data above suggest, it was thought that increasing the time of incubation might lead to equal levels of growth with both amino acids. Data (not shown) show that this did not occur.

CONCLUSIONS

From the results presented, use of *Leuconostoc mesenteroides* for measurement of methionine appears promising, especially when many samples are to be analyzed. The advantages are that (1) many samples (300 per week in our hands) can be analyzed quickly, (2) from cowpea, all methionine is required, (3) mild hydrolysis can be used since complete protein hydrolysis is unnecessary, and (4) methionine derivatives known to be inferior to methionine in fulfilling the methionine requirement in chick are nutritionally inferior to methionine when fed also to this bacterium. The disadvantages are that not all methionine is recovered from protein of at least *P. vulgaris*. Secondly, a potential problem exists since methionine antagonists in the sample will interfere with methionine uptake or utilization. Kihara and Snell (1955) found that ethionine inhibited methionine-dependent growth. Thirdly, cysteine gives only 80% on a weight basis of the growth obtained with cystine. Because of these problems, we suggest that this assay be used for routine assays, as in screening programs, and that samples of particular interest be analyzed by other means.

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