Cystine Content of Proteins, Foods, and Feeds. Comparison of Chromatography on a Sulfonated Polystyrene Resin and Microbiological Methods of Determination

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The cystine contents of a number of proteins, foods, and feeds were determined by chromatography after oxidation to cysteic acid, and by three microbiological assay methods. The microbiological methods used a lanthionized casein medium after 30 minutes of hydrolysis or an oxidized peptone medium after 30 minutes or 6 hours of hydrolysis by autoclaving. The microbiological procedures using 30-minute hydrolysis gave higher values than the chromatographic method, but results were erratic. Most of the cystine values obtained by microbiological assay of the 6-hour digests were lower than the chromatographic values. One commercially isolated soybean protein preparation showed much more cystine by the chromatographic procedure than by microbiological assay, indicating a loss of cystine availability during preparation.

CYSTINE AND METHIONINE are among the amino acids most apt to be deficient in the common foods and feeds of vegetable origin (*11*). Cystine is not considered an essential amino acid, because it can be replaced in the diet by methionine. It is important, however, because it can supply part of the amino acid sulfur required and, thus, spare methionine.

Most values obtained for the cystine content of foods and feeds have been low, because some of the cystine is destroyed during acid hydrolysis in the presence of carbohydrates (*9*).

Recently, two new methods have been described for the determination of cystine in the presence of carbohydrates. Schram, Moore, and Bigwood (*13*) oxidized the protein-bound cystine to cysteic acid and determined the amount of cysteic acid by separation on ion exchange chromatographic columns of Dowex 2 after acid hydrolysis of the protein. Horn and Blum (*8*) determined cystine by microbiological assay, using "lanthionized" casein as a source of amino acids. They hydrolyzed their foodstuffs by refluxing for only 2 hours rather than the regular 24 hours.

The cystine contents of a number of biological materials were determined by chromatographic separation of cystine after conversion to cysteic acid and by three microbiological techniques. Cystine was determined in a number of different types of material, such as seeds, poultry feed ingredients, soybean products, and proteins. The effects of adding sucrose to the protein and autoclaving various proteins on the cystine content determined by four methods were also studied. Chromatographic determination of cystine as cysteic acid was used as the standard method, because cystine is quantitatively oxidized to cysteic acid, which is stable to acid hydrolysis even in the presence of large amounts of carbohydrates (*13*).

Experimental

Cystine was determined chromatographically essentially as described by Schram, Moore, and Bigwood (*13*), with modifications of Bandemer and Evans (*1*). The material to be analyzed was oxidized with performic acid to convert cystine and cysteine to cysteic acid. The oxidized protein was hydrolyzed by heating in the autoclave at 15 pounds' pressure for 6 hours with 20% hydrochloric acid. The hydrochloric acid was removed by evaporation on the steam bath. Water was added twice and evaporation repeated. The solution was made to volume and aliquots were chromatographed on a column of the chloroacetate form of 200- to 400-mesh Dowex 2X10 resin.

Two methods of microbiological assay were used for determination of cystine. *Leuconostoc mesenteroides* P-60 was the organism in both methods. The first method, the peptone medium method, used the medium of Lyman *et al.* (*10*), which contained oxidized peptone as the source of most of the amino acids. The second method, the lanthionized casein medium method, used the medium of Horn and Blum (*8*), which contained lanthionized casein hydrolyzate as the source of most of the amino acids.

Acid hydrolyzates were prepared by autoclaving 1.0 gram of the material with 20 ml. of 20% hydrochloric acid at 15 pounds' pressure for 30 minutes or 6 hours. The 6-hour hydrolysis has been used in this laboratory for some time in preparing hydrolyzates for microbiological assay of most of the amino acids. The 30-minute hydrolysis was chosen as a result of the following experiments.

One-gram samples of soybean oil meal were weighed into 125-ml. Erlenmeyer flasks, and 20 ml. of 20% hydrochloric acid was added. Duplicate samples were then autoclaved at 15 pounds' pressure for 15, 30, 45, or 60 minutes. The same soybean oil meal was used in a duplicate second experiment, in which samples were also autoclaved for 120 minutes. All samples were assayed for cystine, using both peptone and lanthionized casein media (Table I).

Sixty-one samples were analyzed by the chromatographic procedure. Cystine was also determined on most of these same samples by microbiological assay after 30 minutes of hydrolysis, using both the oxidized peptone and lanthionized casein media. Some samples were assayed microbiologically for cystine after 6 hours of hydrolysis, using the oxidized peptone medium.

Results and Discussion

The results of the hydrolysis studies were not conclusive because low values

were obtained with the peptone medium in the second experiment. Repeat assays gave consistently low values, showing the trouble to be in the digestion procedure (Table I). The 30-minute period was chosen for routine assays because it gave the highest values most often.

The question might be raised as to the reason for the differences in results obtained in experiments 1 and 2 of Table I. During hydrolysis of the soybean oil meal two reactions are taking place: the release of cystine, or of peptides that contain cystine in a form available for the assay organism; and reaction of cystine with carbohydrate to cause destruction of the cystine. The largest value is obtained where the curves of the two reactions intersect. Slight differences in time or conditions of hydrolysis will shift the point of intersection, or the highest point may be somewhere between two of the hydrolysis times used. The data in Table I indicate the possibility of erratic results from the use of short hydrolysis time for microbiological assay for cystine.

Nine different seeds, both raw and heated, were analyzed by the chromatographic and microbiological procedures, using a 30-minute hydrolysis and both oxidized peptone and lanthionized casein media (Table II). The results agree fairly well for the most part, except that the lanthionized casein medium gave slightly high results on some samples.

Several poultry feed ingredients and mashes were analyzed by all four methods (Table III). The chromatographic values were generally lower than the microbiological assays using 30-minute digests and higher than those using 6-hour digests.

Cystine was determined on several commercially available isolated soybean proteins as obtained, and after autoclaving alone or in a mixture of 80% protein and 20% sucrose (Table IV). The cystine contents of soybean oil meal and four of the five soybean proteins were nearly the same whether determined chromatographically or microbiologically after 30 minutes of hydrolysis using the peptone medium. The lanthionized casein medium gave higher values in most cases than either the peptone medium or the chromatographic method. The microbiological procedures consistently gave low values for one of the five isolated soybean protein preparations (Alpha protein) when compared with the chromatographic determination. The microbiological assay procedure gave values similar to or higher than those of the chromatographic method for the isolated milk protein, casein, and the isolated corn protein, zein (Table V).

Schram, Moore, and Bigwood (*13*) found that the chromatographic cystine determination is specific for cystine and cysteine because both are oxidized quantitatively to cysteic acid, and cysteic acid is not destroyed during acid hydrolysis even in the presence of large amounts of carbohydrates. Horn and Blum assumed that because the short hydrolysis time gave highest results for cystine, these are necessarily correct results. They considered the possibility of peptide stimulation, but dismissed it because peptides did not stimulate other amino acid assays. However, Evans and McGinnis (*6*) and Evans and St. John (*7*) observed that cystine peptides exerted a greater stimulating action on growth of *Lactobacillus arabinosus* on a cystine-free medium than did free cystine.

Table I. Effect of Time of Hydrolysis on Cystine Liberated from Soybean Oil Meal by Hydrochloric Acid

	Microbiological Assay, %			
	Peptone Medium		Lanthionized Casein Medium	
Time, Min.	Expt. 1	Expt. 2	Expt. 1	Expt. 2
15	1.04	0.71	0.93	0.90
30	0.98	0.74	0.95	1.01
45	0.96	0.81	0.87	0.95
60	0.88	0.76	0.80	0.88
120	...	0.63	...	0.78

Experiment 1 was conducted about a month before experiment 2, but portions of the same sample of soybean oil meal were used for each. The peptone and lanthionized casein medium assays were performed on aliquots of the same hydrolyzates.

Table II. Cystine Found in Seeds

(Calculated to basis of 16% nitrogen)

			30-Min. Hydrolysis, %	
Material[a]	% N	Chromatographic, %	Peptone medium	Lanthionized casein medium
Barley	1.53	2.3	2.1	2.7
Barley heated	1.60	1.9	2.0	2.5
Mung bean	3.89	0.6	0.7	0.6
Mung bean heated	3.99	0.5	0.6	0.6
Pea bean	3.84	1.1	0.6	1.2
Pea bean heated	3.84	1.1	0.5	1.2
Corn a[a]	1.73	2.0	1.8	2.4
Corn a heated	1.73	1.9	2.0	2.4
Millet	2.24	1.7	0.9	1.9
Millet heated	2.28	1.3	0.9	2.0
Rice	1.05	1.5	1.8	2.1
Rice heated	1.15	1.4	1.7	2.1
Sudan grass seed	1.65	1.4	1.5	1.4
Sudan grass seed heated	1.75	1.2	1.5	1.5
Sunflower seed	4.95	1.6	1.9	2.3
Sunflower seed heated	4.80	1.3	1.3	1.1
Wheat a	1.59	2.1	2.3	2.8
Wheat a heated	1.62	2.0	2.2	2.5

[a] Name of sample followed by a letter indicates that different samples were analyzed and data for each are given. Materials were obtained from various sources, and those obtained from any one source are grouped together.

Table III. Cystine Found in Poultry Feed Ingredients

(Calculated to basis of 16% nitrogen)

			30-Min. Hydrolysis, %		6-Hr. Hydrolysis, %,
Material[a]	% N	Chromatographic, %	Peptone medium	Lanthionized casein medium	Peptone Medium
Alfalfa meal	3.02	0.9	0.7	0.8	0.4
Dried buttermilk	4.92	0.7	1.0	1.5	0.8
Chick starter mash	3.55	1.3	1.6	1.9	0.9
Corn b	1.64	1.6	1.9	1.8	1.4
Corn fermentation solubles	4.11	2.3	...	...	...
Cottonseed meal	6.64	2.2	1.7	1.8	...
Fish meal	9.66	0.7	1.1	1.3	0.7
Fish meal (menhaden)	9.91	0.7	...	...	...
Oats	1.87	2.2	2.9	3.0	1.8
Poultry mash	3.18	1.3	1.7	2.0	1.0
Soybean oil meal a	8.34	1.2	1.5	1.7	0.9
Soybean oil meal b	7.90	1.2	1.3	1.4	...
Wheat b	1.88	1.9	2.1	2.2	1.4
Wheat bran	2.29	1.3	2.3	2.5	1.2
Wheat middlings	2.65	1.4	1.9	1.9	1.1
Whey	6.63	0.6	...	...	...

[a] See footnote, Table II.

Table IV. Cystine Found in Soybean Oil Meal and Some Isolated Soybean Proteins

(Calculated to basis of 16% nitrogen)

Material[a]	% N	Chromatographic, %	30-Min. Hydrolysis, % Peptone medium	30-Min. Hydrolysis, % Lanthionized casein medium	6-Hr. Hydrolysis, % Peptone Medium
Soybean oil meal c	7.90	1.54	1.60	2.07	1.14
Soybean oil meal c autoclaved	8.24	0.85	0.82	0.87	0.60
Alpha protein a[b]	14.39	0.69	0.29	0.31	0.24
Alpha protein a autoclaved	14.37	0.65	0.26	0.27	0.20
Alpha protein a + sucrose	11.63	0.59	0.30	0.18	0.17
Alpha protein a + sucrose autoclaved	12.20	0.59	0.26	0.20	0.17
Alpha protein b	14.39	0.66	0.34	0.33	...
Amisoy[b]	13.51	1.30	1.37	1.91	0.78
Amisoy acid washed	12.38	1.52	...	...	...
Buckeye protein a[b]	14.32	1.24	1.38	1.72	0.85
Buckeye protein a autoclaved	14.35	0.81	1.00	0.99	0.67
Buckeye a + sucrose	11.45	1.22	1.37	1.56	0.88
Buckeye protein a + sucrose autoclaved	12.12	0.95	0.82	1.03	0.73
Buckeye protein b	14.32	1.18	1.52	1.56	...
C-1 assay protein[b]	13.75	1.22	1.08	1.40	0.70
Protosoy[b]	13.64	1.14	1.10	1.46	0.70

[a] See footnote, Table II.

[b] Alpha, Buckeye, Protosoy, Amisoy, and C-1 assay are commercial isolated soybean proteins prepared by Glidden Co. (now Central Soya Co.), Buckeye Cellulose Corp., Mann Research Labs., Glidden Co., and Dracket Co. (now Archer-Daniels-Midland Co.), respectively.

Table V. Cystine Found in Proteins

(Calculated to basis of 16% nitrogen)

Material	% N	Chromatographic, %	30-Min. Hydrolysis, % Peptone medium	30-Min. Hydrolysis, % Lanthionized casein medium	6-Hr. Hydrolysis, % Peptone Medium
Casein	13.46	0.37	0.34	0.43	0.33
Casein autoclaved	13.34	0.31	...	...	0.28
Casein + sucrose	10.92	0.32	...	...	0.47
Casein + sucrose autoclaved	11.32	0.25	...	...	0.44
Zein	14.96	0.49	0.60	0.67	0.49
Zein autoclaved	14.96	0.42	...	...	0.38
Zein + sucrose	12.15	0.45	...	...	0.38
Zein + sucrose autoclaved	12.12	0.36	...	...	0.34
Dried whole egg (defatted)	11.64	2.34	...	...	2.89
Egg white	13.23	2.61	...	...	3.02
Egg yolk (defatted)	14.28	1.69	...	...	1.90

The higher values for cystine obtained by the microbiological procedures using 30-minute hydrolysis than by the chromatographic method appear to be better explained by a stimulating effect of cystine peptides on assay bacteria than by a loss of cystine in the chromatographic procedure.

Evans, Groschke, and Butts (*5*) observed a loss of 31% of the cystine from soybean oil meal that had been autoclaved for 4 hours at 15 pounds' pressure. None of the cystine of isolated soybean protein (Alpha protein) was destroyed when the protein was autoclaved either alone or as a mixture of 80% protein and 20% sucrose, glucose, dextrin, agar-agar, gum arabic, or soybean oil. Cystine was determined by assay with *L. mesenteroides* after hydrolysis by autoclaving with 20% hydrochloric acid for 6 hours. The data in Table IV confirm the results of Evans *et al.* (*5*), except that over 40% destruction of cystine occurred in autoclaved soybean oil meal. Contrary to the results with Alpha protein, autoclaving destroyed 27 to 42% of the cystine in Buckeye protein (depending upon method of determination). No further destruction took place when the protein was mixed with 20% of sucrose. Assuming that pure soybean protein contains 16% nitrogen, Buckeye protein contained 11% of impurities. These may be the cause of the cystine destruction when Buckeye protein was autoclaved.

Some destruction of cystine also occurred when zein or casein was autoclaved, and the addition of sucrose increased the destruction very little (Table V).

Alpha protein differed from the rest of the isolated soybean proteins in that it contained less cystine. The proteins (N × 6.25) of soybean oil meal contained 1.16 to 1.54% cystine by chromatographic analysis and 1.34 to 2.06% by microbiological assay after 30-minute hydrolysis. About half of the cystine was lost from the total soybean protein in preparation of Alpha protein according to chromatographic determination. When microbiological assay was used, at least 80% was lost or was inactivated so that it was not available for *L. mesenteroides* P-60. A cystine-rich portion of soybean proteins is, therefore, not included in the Alpha protein fraction. Van Etten *et al.* (*15*) found a higher concentration of cystine in heat-coagulated soybean protein than in the acid-precipitated protein.

Alpha and Buckeye proteins must be different fractions of the total soybean proteins. Buckeye protein contains the soybean trypsin inhibitor. Alpha protein does not. Buckeye protein contains about twice as much cystine as does Alpha protein determined chromatographically, and five times as much by microbiological assay after 30 minutes of hydrolysis.

Three possible explanations for the peculiar behavior of Alpha protein are apparent from the data. The first is that in the method of preparing Alpha protein, considerable cystine is oxidized to cysteic acid or converted to some form that is oxidized to cysteic acid by the method used in the chromatographic study. Cysteic acid or the other compound is not available to *L. mesenteroides* P-60. Another possible explanation is that the cystine in Alpha protein is combined with other amino acids in such a way that it is liberated from the protein more slowly than from the other soybean proteins. The amount of free cystine present in the hydrolyzate would then be small, because it would be destroyed almost as fast as it is liberated. The third explanation is also connected with the possibility of a different type of cystine binding in Alpha protein than in other soybean proteins. It is possible that some cystine peptides are more active for the microbiological assay than others and that the peptides of Alpha protein that are liberated by autoclaving with 20% hydrochloric acid for 30 minutes are not as active for the growth of *L. mesenteroides* as those liberated from soybean oil meal or Buckeye protein. The first explanation appears to the authors to be the most plausible, but there is evidence for the last, in that microbiological assay of the other soybean proteins using the lanthionized casein medium gave higher values than the chromatographic procedure, indicating stimulation of growth by something other than cystine.

The colorimetric method of Sullivan (*14*) has been the most specific chemical procedure for the determination of cystine, and most of the earlier data in the literature were obtained by this procedure. It, however, suffers from the limitation that the cystine has to be

first liberated from the proteins by acid hydrolysis. The colorimetric method was not used in the present study, because losses of cystine during prolonged acid hydrolysis of grains and other carbohydrate-containing materials would be expected (*9*). Sarkar, Luecke, Evans, and Duncan (*12*) compared the microbiological with the colorimetric method and obtained similar values by the two methods. They used aliquots of the same hydrolyzates for microbiological and chemical methods. They first precipitated cystine from the acid hydrolyzates as the cuprous mercaptide by the procedure of Csonka, Lichtenstein, and Denton (*2*) in the chemical procedure, and determined cystine by the colorimetric Sullivan reaction as modified by Evans (*4*). Values obtained by Sarkar *et al.* (*12*) are compared in Table VI with some of those from Tables II to V.

Evans (*3*) used a differential oxidation procedure for the determination of cystine and methionine in plant and animal materials. The method assumes that all of the organic sulfur oxidized to sulfate by concentrated nitric acid is cystine or cysteine sulfur. High results will be obtained if other easily oxidizable organic sulfur compounds are present. A comparison of some of the data obtained by the differential oxidation procedure (*3*) with those of Tables II to V is of interest (Table VI). The differential oxidation procedure gave slightly higher values for most of the materials listed except barley. Fish meal showed much more cystine by the differential oxidation procedure than by the other methods, but this was no doubt caused by differences in the fish meals studied. For the most part, the differential oxidation procedure gave values very close to the chromatographic and microbiological ones, even though analyses were not made on the same samples.

The data of Horn and Blum (*8*) agree well with those presented in this paper, except for wheat, zein, and whole egg, which are much higher. The values of Sarkar *et al.* (*12*) are low for cystine in zein.

Table VI. Comparison of Several Methods for the Determination of Cystine

(Per cent cystine. Calculated to a basis of 16% nitrogen)

Material	Chromatographic	Microbiological				Chemical (Sarkar) (12)	Differential Oxidation (3)
		30 min.	6 hr.	Horn (8)	Sarkar (12)		
Alfalfa	0.9	0.7	0.4	...	...	...	1.8
Barley	2.3	2.1	...	2.0	...	...	1.7
Casein	0.37	0.34	0.33	0.38	0.23	0.26	0.5
Corn	2.0	1.8	1.4	2.5	...	...	2.3
Cottonseed meal	2.2	1.7	...	1.7	...	...	2.4
Whole egg (defatted)	2.3	...	2.9	2.9	...	...	...
Fish meal	0.7	1.1	0.7	...	...	...	1.8
Oats	2.2	2.9	1.8	3.0	...	...	3.0
Rice	1.5	1.8	...	1.8	...	...	...
Soybean oil meal	1.3	1.5	1.0	1.9	...	...	2.0
Wheat	2.2	2.4	1.7	2.2	...	...	2.6
Wheat middlings	1.4	1.9	1.1	...	...	...	2.2
Whey	0.6	...	...	...	...	...	2.6
Zein	0.5	0.6	0.5	1.4	0.20	0.20	...

Summary

The cystine contents of a number of proteins, foods, and feeds were determined by chromatography and three microbiological procedures. Chromatography of cysteic acid gave the most reproducible results, was considered most satisfactory, and was used as the standard of comparison. Chromatography generally gave lower values than the two microbiological assay procedures using a 30-minute hydrolysis period, and the microbiological procedures gave erratic results. Most of the cystine values obtained by microbiological assay of the 6-hour digests were lower. The chromatographic procedure showed more cystine in one commercially isolated soybean protein preparation than microbiological assay, indicating a loss of cystine availability during preparation of the protein or presence of a bacterial growth inhibitor in the protein.

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