Optimized Determination of Cystine/Cysteine and Acid-Stable Amino Acids from a Single Hydrolysate of Casein- and Sorghum-Based Diet and Digesta Samples

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A facile method for simultaneously derivatizing cyst(e)ine with 3,3′-dithiodipropionic acid (DTDPA) to the mixed disulfide (Cys-X) and hydrolyzing protein prior to amino acid analysis was applied to complex samples consisting of diets and digesta from an animal feeding study. Modified chromatographic conditions allowed adequate separation of all acid-stable amino acids in 90 min with Cys-X eluting between valine and methionine. Repeated chromatography of these hydrolysates led to loss of column performance, which could be reversed by back-flushing the column with a high pH/ion strength regenerant. Passing hydrolysates through a cation exchange cleanup column removed the interfering compound and allowed quantitative recovery of all amino acids. The conversion of cyst(e)ine to Cys-X increased as the molar ratio of DTDPA to cystine increased from 2.6 to 211. Hydrolysis times >75 min had no effect on production of Cys-X. Total amino acid yield from hydrolysates increased with time up to 540 min for most samples, and recovery was higher from diets than from digesta. Coefficients for correcting amino acid yield as a function of hydrolysis time were derived.

Keywords: *Cystine; cysteine; amino acid analysis; 3,3′-dithiodipropionic acid*

INTRODUCTION

Amino acid analysis is a necessary component of many approaches to determining protein nutritional quality. Although a number of precolumn derivatization techniques have been developed (Bidlingmeyer et al., 1987; Cohen and De Antonis, 1994), the most utilized chromatographic technique for separating and quantifying amino acids from hydrolysates of food and related samples remains ion exchange chromatography and postcolumn reaction with ninhydrin to produce a chromophore quantifiable by visible spectroscopy (Elkin and Griffith, 1985). The method used for the hydrolysis of protein prior to amino acid analysis is of considerable importance because specific amino acids are released and destroyed at differing rates depending on hydrolysis conditions. Probably the most used method for acid hydrolysis is still that of Moore and Stein (1963), who reported that the best "all around" hydrolysis can be achieved by reaction for 24 h with 6 N HCl at 110 °C under conditions rigorously excluding oxygen, nonprotein substances, and metals. Unfortunately, it is not always possible to exclude non-protein species when one is dealing with foods, feeds, and similar complex samples.

The rates of decomposition of the amino acids during hydrochloric acid hydrolysis are dependent on several factors including the following: the concentration of the hydrolyzing acid; the purity of the acid used; the time and temperature of hydrolysis; and the presence of carbohydrates, aldehydes, or metal impurities (Roach and Gehrke, 1970). Roach and Gehrke (1970) reported that the maximum yield for all of the protein amino acids was obtained at 145 °C for 4 h, which was in good agreement with hydrolysis at 110 °C for 24 h. Phillips (1983) developed an accelerated procedure for preparing acid hydrolysates of complex proteins and reported that hydrolysis at 145 °C for 1.25 h produced results comparable for the different schemes. Generally, acid hydrolysis will yield over 95% for most amino acids. However, tryptophan is completely destroyed and a considerable loss of cyst(e)ine may occur, whereas 5−15% of threonine and serine are destroyed (Roach and Gehrke, 1970). Isoleucine and valine are liberated more slowly than the other amino acids. An accurate estimate of the true isoleucine and valine content can be obtained by amino acid analysis of samples hydrolyzed for various periods and then calculation of the data to infinite time. Also, an extrapolation to "zero time" can be made to determine more accurate values for those amino acids that undergo serious decomposition, i.e., threonine and serine (Blackburn, 1978). The loss of tyrosine through formation of 3-chlorotyrosine or 3-bromotyrosine can be prevented by addition of phenol and/or mercaptoethanol to the hydrolysis acid. The inclusion of phenol in hydrolysis mixtures protected other labile amino acids such as phenylalanine, histidine, and arginine as well (Blackburn, 1978; Gehrke et al., 1985).

A number of methods for recovering sulfur-containing amino acids from proteins have been reported. The instability of cystine and cysteine during acid hydrolysis can be overcome by prior oxidation with performic acid of cystine and cysteine to cysteic acid, which is stable under acid hydrolysis conditions (Moore, 1963). The preoxidation was extended to permit the accurate determination of methionine as the sulfone. Inglis and Liu (1970) reported the recovery of *S*-sulfocysteine from proteins treated with dithiothreitol and sodium tetrathionate.

Chiou and Wang (1988) reported that high-tempera-

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ture hydrolysis (microwave irradiation at >150 °C) with 4 M methanesulfonic acid can be successfully applied to the amino acid analysis of peptides and proteins. All amino acids, including cystine and tryptophan, were accurately recovered from a single 45 min hydrolysate. Yamada et al. (1991) reported good recoveries of all amino acids including cystine and tryptophan when microquantities of protein were modified by vapor-phase *S*-pyridylethylation and hydrolysis in methanesulfonic acid vapors for as little as 12.5 min. Hale et al. (1996) reported the use of volatile reagents to reduce (triethylphosphine) and alkylate (bromopropane) cysteine residues prior to hydrolysis. Recently, Strydom and Cohen (1993) reported the combination of 3,3′-dithiodipropionic acid (DTDPA) derivatization/vapor-phase hydrolysis with posthydrolysis derivitization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate applied to several pure proteins.

In the present study, the method of Barkholt and Jensen (1989) was investigated as a way of recovering all amino acid residues except tryptophan from a single hydrolysate. This approach involves hydrolysis of proteins in 6 N HCl with phenol containing DTDPA. Cyst(e)ine is reported to be quantitatively converted to the mixed disulfide (Cys-X):

$$HOOC-CH_2-CH_2-S-S-CH_2CHNH_2COOH$$

which can be separated from other amino acid residues by ion exchange chromatography. In addition to verifying that this method could be successfully applied to free cyst(e)ine and pure proteins in our system, our goal was to demonstrate that it is useful for complex samples as well.

This study developed a method for removing the compound(s) that degrade column performance from hydrolysates, maximizing the yield of Cys-X, and optimizing the HPLC conditions for separating and quantifying Cys-X. The effect of hydrolysis time on the amino acid profile of diet and digesta samples was examined under the optimized DTDPA and cleanup procedure.

MATERIALS AND METHODS

Acid Hydrolysis Conditions for DTDPA−Cystine Reaction. Ten milliliters of 6 N HCl (1:2 dilution of ACS Plus grade, Fisher Scientific, Norcross, GA) containing 0.5% phenol (Fisher, Certified ACS) was added to various amounts (0.05−1.0 mL) of 9 μm/mL DL-cystine (Sigma Chemical Co., St. Louis, MO) solution in heavy wall glass hydrolysis tubes. These were equipped with stopcock-fitted tops which could be clamped into place such that the tubes could be sealed under either inert gas (Zero Grade argon, Air Products Co., Columbus, GA) or vacuum. Aliquots (0.25−1 mL) of 1 or 2% DTDPA (Aldrich Chemical Co., Milwaukee, WI) in 0.2 M NaOH in were added to samples to convert cystine/cysteine to a stable derivative during acid hydrolysis as modified from the method of Barkholt and Jensen (1989). Molar ratios of DTDPA/cystine between 2.6 and 211 were studied. Samples were deaerated by three cycles of alternate evacuation and argon purging and then sealed under vacuum. Hydrolysis was carried out at 145 °C (Phillips, 1983) in a heating block (Model BD-40, Technicon, Tarrytown, NY) with occasional shaking. Effects of hydrolysis times of 45, 75, 240, and 540 min on the conversion of cystine to Cys-X were studied. Hydrolysates were pH adjusted to 1.6, diluted to 25 mL, and filtered through 0.2 μm Teflon filters (Millipore Corp., Bedford, MA). Alternatively, hydrolysates were pH adjusted to 7.5 in preparation for passing through a cleanup column.

Acid Hydrolysis Conditions for Protein−DTDPA Reaction. Pure protein and complex protein samples were used to test the effectiveness of DTDPA in converting cystine/cysteine to Cys-X, as well as to test effects of the cleanup procedure on the overall recovery of amino acids. Samples, including lysozyme from chicken egg white (Sigma), 10% protein diets containing casein or sorghum grain, and pig ileal digesta arising from the two diets, were hydrolyzed with and without DTDPA. Both diet samples contained 5% fat (endogenous fat plus cottonseed oil), 0.2% vitamin mix, 0.1% mineral mix, and 0.25% chromic oxide as an unabsorbed marker. The sorghum diet contained, on a dry basis, 88.5% whole ground sorghum and 6.2% of an equal weight mixture of dextrose and cornstarch in addition to fat, vitamins, and minerals. The casein diet contained, on a dry weight basis 10.9 g of casein (Sigma), 89 g of starch plus dextrose, and 3.2% cellulose in addition to fat, vitamins, and minerals. Samples containing ~10 mg of protein were weighed into hydrolysis tubes. To these were added 10 mL of 6 N HCl containing 0.5% phenol and 0.5 mL of 9 μm/mL norleucine (Sigma) as an internal standard. The same samples were subjected to reaction with DTDPA. Three milliliters of 2% DTDPA/0.2 M NaOH, 5 mL of 12 N HCl, 2 mL of 2.5% phenol, and 0.5 mL of 9 μm/mL norleucine were added to samples containing 10 mg of protein to give a molar ratio of DTDPA/Cys2 >100. All samples were deaerated as described above and sealed under vacuum for hydrolysis at 145 °C for 75 min. After dilution, pH adjustment, and filtration, hydrolysates were ready for HPLC injection or cleanup procedure. Cys-X standards were prepared by heating cystine with DTDPA under the same conditions as used for hydrolysis.

Cleanup Procedure for Cys-X-Containing Hydrolysates. Hydrolysates were adjusted to pH 7.5 and made to volume in 25 mL volumetric flasks. Ten milliliters of each hydrolysate was loaded on a "cleanup" column containing AG 50W-X12 analytical grade cation exchange resin (Bio-Rad Laboratories, Richmond, CA) equilibrated with sodium citrate−NaCl buffer (1.2 M Na, 0.1 M citrate) at pH 7.5. Cleanup column bed volumes of 1, 3, 5, and 10 mL were examined to find conditions under which all amino acids could be quantitatively recovered without requiring excessive amounts of buffer. Each sample was eluted with 30 mL of the pH 7.5 sodium citrate−NaCl buffer. Collected eluants were pH adjusted to 1.6, made to 50 mL volume, and then filtered through 0.2 μm Teflon filters prior to amino acid analysis.

Amino Acid Analysis by Cation Exchange HPLC. The amino acid composition of the hydrolysate of each sample was determined by ion exchange chromatography using Waters HPLC components and Baseline 810 chromatography workstation software (Millipore Corp., Milford, MA). The system was equipped with a Waters System Interface Module (SIM), two Waters Model 510 HPLC pumps (for buffers), a Waters Intelligent Sample Processor (WISP) Model 710B, an Eldex reagent pump delivering 0.3 mL/min of ninhydrin, a Waters temperature control module, a postcolumn reaction coil (9 m in length) in a coil oven, a column oven, and a Waters Model 440 absorbance detector. The cation exchange amino acid analysis column was from Pickering Laboratories (Mountain View, CA). Ninhydrin (Trione, Pickering) was used for the postcolumn derivatization of amino acids at a reaction coil temperature of 120 °C, and reaction products were detected at 436 and 546 nm. Amino acids in unknowns were quantified using a standard mixture of amino acids containing the acid-stable amino acids (P/N 012506H, Pickering).

To separate Cys-X from other amino acids, it was necessary to vary column temperature and buffer gradient from the usual conditions. Amino acid elution patterns were investigated using gradients that were isocratic in 100% buffer A [0.2 N Na buffer, pH 3.28 (Pickering)] for 8, 10, or 12 min and then changed linearly to 100% buffer B [1.0 N Na buffer, pH 7.40 (Pickering)] at 24, 30, 32, 34, and 35 min, at a total flow rate of 0.3 mL/min.. The effects of column temperatures of 46, 48, 50, and 52 °C were also investigated. Tryptophan was not determined.

Optimized Conditions−Effect of Hydrolysis Time on Amino Acid Profile. The effect of hydrolysis time on amino acid recovery/profile was determined using complex samples−diets containing 10% protein from casein or whole, ground

Table 1. Effect of Reaction Time and DTDPA/Cystine Mole Ratio on Recovery of Cys-X Heated in 6 N HCl/0.5% Phenol at 145 °C

cleanup column	hydrolysis time (min)	mole ratio DTDPA/cystine	Cys-X recovery %[a]
no	45	2.6	77.5
no	45	5.3	85.9
no	45	26.4	85.8
no	75	2.6	84.6
no	75	5.3	89.8
no	75	26.4	92.2
	240	2.6	81.1
no	240	5.3	88.7
no	240	26.4	94.5
yes	75	26.4	97.4
yes	75	52.8	98.3
yes	75	105.6	99.1
yes	75	211.1	100.0

[a] Mean results from duplicate reactions and chromatograms. Recovery % = (peak area of Cys-X) × 100/(peak area of Cys + peak area of Cys-X).

sorghum and the corresponding ileal digesta (freeze-dried) from swine. Samples were hydrolyzed as previously described in 6 N HCl/0.5% phenol and DTDPA for hydrolysis times of 50, 75, 240, and 540 min at 145 °C. Hydrolysates were pH adjusted to 7.5 and filtered through Whatman No. 1 filter papers. Ten milliliters of the eluants was passed through a 3 mL bed volume cleanup column and eluted with 30 mL of 1.2 M sodium buffer at pH 7.5 as described above. Amino acid content was determined under the optimal chromatographic conditions—column temperature of 46 °C and gradient program that was isocratic in buffer A for 10 min, changed linearly to 100% buffer B from 10 to 30 min, and remained isocratic for the remainder of the 90 min run.

RESULTS AND DISCUSSION

Customary conditions for hydrolysis of proteins in this laboratory involve heating samples in 6 N HCl/0.5% phenol for 75 min at 145 °C. The effects of hydrolysis/reaction time at 145 °C and molar ratio of DTDPA to cystine on conversion of free cystine to Cys-X are shown in Table 1. Slightly lower yields were observed at 45 min of reaction time than at 75 min, but extending the time from 75 to 240 min produced no further increase. A small amount of residual cystine was present in chromatograms at lower reagent to cystine ratios, but it disappeared when the molar ratio was 105. Yields of Cys-X increased with DTDPA to cystine ratio. Conditions selected for further investigation were 75 min of hydrolysis time and a DTDPA/cystine ratio of ≥100, which is comparable to the ratio (∼120) used by Barkholt and Jensen (1989). The application of the cleanup procedure did not affect the recovery of Cys-X, but it did protect the analytical column from the interfering compound in the DTDPA-treated hydrolysates. It should be noted that without knowing the relative response factors for Cys-X compared to cystine, these recoveries should be interpreted as approximate.

Usual conditions for separating amino acids on the Pickering column used in our laboratory include a column temperature of 50 °C and a buffer gradient that is isocratic at 100% buffer A (0.2 N Na buffer, pH 3.28) for the first 10 min, then changes linearly to become 100% buffer B (1.0 N Na buffer, pH 7.40) from 10 to 32 min, and remains isocratic in buffer B until a total of 90 min have elapsed. Under these conditions, Cys-X coeluted with valine. Lowering the column temperature to 46 °C and steepening the gradient slightly to one in

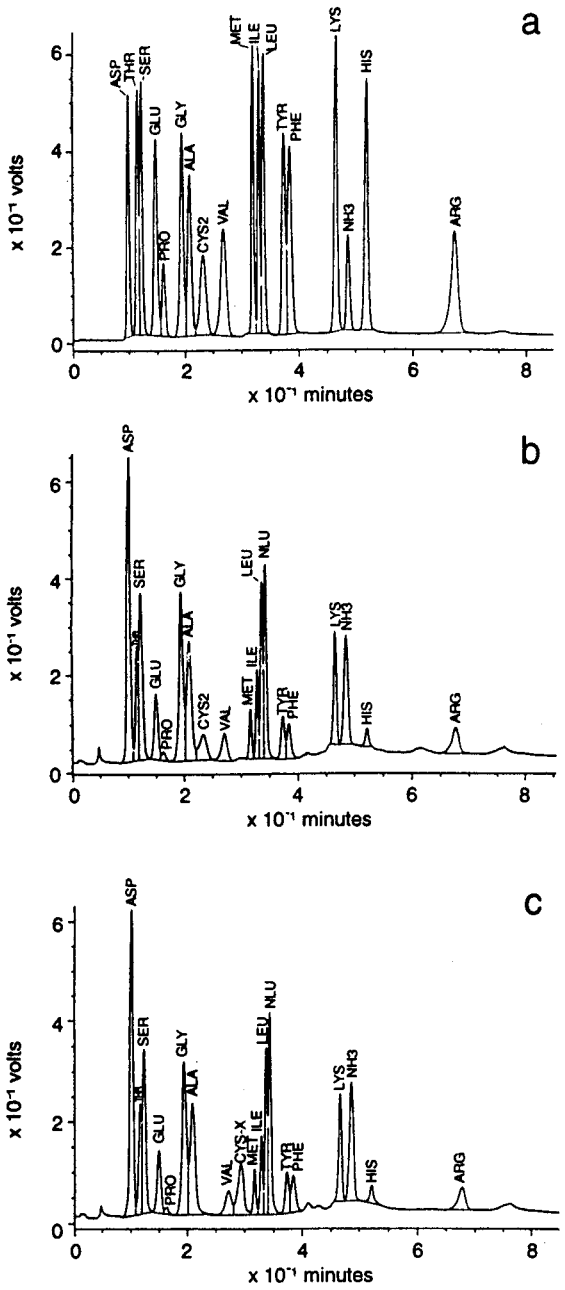

Figure 1. Chromatograms of amino acids: (a) amino acid standard chromatographed under modified conditions [column temperature = 46 °C; linear gradient, 100% buffer A (0.2 N Na, pH 3.28) to 100% buffer B (1.0 N Na, pH 7.40) 10−30 min]; (b) hydrolysate of lysozyme without DTDPA under the same conditions as for (a) (c) hydrolysate of lysozyme with DTDPA under the same conditions as for (a).

which the linear gradient began at 10 min and ended at 30 min produced adequate separation of a standard amino acid mixture (Figure 1a) and of a hydrolysate of lysozyme without DTDPA (Figure 1b), where cystine eluted between alanine and valine at ∼24 min. It also allowed resolution of Cys-X from the other amino acids as shown for lysozyme hydrolyzed in the presence of DTDPA as shown in Figure 1c, where the cystine peak disappeared and the Cys-X peak appeared between valine and methionine at ∼29 min. Better separation between glycine and alanine, glutamate and proline, and tyrosine and phenylalanine was observed at higher temperature (52 °C), while the separation between threonine and serine required lower temperature (46 °C). The sharper gradient also helped the separation of tyrosine and phenylalanine. The chromatograpic

Table 2. Amino Acid Composition (Grams of Amino Acid per 100 g of Total Amino Acid) of Lysozyme As Affected by DTDPA Reaction and Cleanup Procedure[a]

		treatments					
		with DTDPA with column		w/o DTDPA with column		w/o DTDPA w/o column	
amino acid	literature value[b]	LYA	ratio[c]	LYB	ratio	LYC	ratio
Asp	18.15	19.38	1.07	19.70	1.09	20.02	1.10
Thr	5.41	5.61	1.04	5.78	1.07	5.61	1.04
Ser	6.82	7.46	1.09	7.46	1.09	7.85	1.15
glu	4.78	4.63	0.97	4.69	0.98	4.69	0.98
Pro	1.49	1.45	0.97	1.34	0.90	1.41	0.94
Gly	5.85	6.57	1.12	6.53	1.12	6.50	1.11
Ala	6.94	7.29	1.05	7.65	1.10	7.86	1.13
Cys	6.23	0	0	5.18	0.83	4.80	0.77
Val	4.56	3.06	0.67	3.19	0.70	2.94	0.64
Cys-X	0	6.50	1.04[d]	0	0	0	0
Met	1.94	2.33	1.20	2.28	1.18	2.36	1.22
Ile	5.11	4.04	0.79	4.21	0.82	4.12	0.81
Leu	6.82	7.01	1.03	7.14	1.05	7.21	1.06
Tyr	3.53	3.85	1.09	3.79	1.07	3.93	1.11
Phe	3.22	3.45	1.07	3.08	0.96	3.23	1.00
Lys	5.70	5.17	0.91	5.38	0.95	5.24	0.92
His	1.01	1.01	1.00	1.13	1.12	1.11	1.10
Arg	12.43	11.18	0.90	11.46	0.92	11.14	0.90

[a] Mean values from hydrolysis and chromatography of duplicate samples. [b] Literature value obtained from Canfield and Liu (1965). [c] Ratio was calculated as the experiment value divided by the literature value. [d] Grams of Cys-X derived from conversion of cystine standard to Cys-X and the ratio was compared with Cys in literature value.

Table 3. Amino Acid Profiles (Grams per 100 g of Sample) of Sorghum Diet and Digesta as a Function of Hydrolysis Time[a]

	sorghum diet, hydrolysis time of				sorghum digesta, hydrolysis time of			
amino acid	50 min	75 min	240 min	540 min	50 min	75 min	240 min	540 min
Asp	0.600	0.664	0.644	0.652	1.203	1.362	1.257	1.228
Thr	0.181	0.256	0.282	0.272	0.560	0.693	0.677	0.644
Ser	0.452	0.483	0.460	0.474	0.817	0.910	0.813	0.787
Glu	1.655	1.843	1.832	1.825	2.090	2.365	2.214	2.237
Pro	0.625	0.716	0.748	0.768	0.921	1.097	1.064	1.087
Gly	0.344	0.323	0.297	0.320	1.153	1.215	1.118	1.126
Ala	0.813	0.893	0.860	0.871	1.165	1.316	1.174	1.131
Val	0.194	0.296	0.377	0.443	0.402	0.537	0.655	0.670
Cys-X	0.220	0.276	0.179	0.236	0.474	0.531	1.286	0.463
Met	0.099	0.175	0.144	0.168	0.385	0.378	0.320	0.290
Ile	0.187	0.256	0.329	0.369	0.581	0.663	0.712	0.652
Leu	0.957	1.108	1.162	1.163	1.296	1.485	1.397	1.371
Tyr	0.343	0.362	0.357	0.362	0.455	0.534	0.498	0.483
Phe	0.380	0.430	0.445	0.489	0.490	0.533	0.566	0.566
Lys	0.134	0.149	0.165	0.193	0.413	0.506	0.546	0.541
His	0.142	0.169	0.179	0.197	0.239	0.310	0.324	0.343
Arg	0.234	0.284	0.320	0.315	0.258	0.438	0.363	0.430

[a] Mean values from hydrolysis and chromatography of duplicate samples.

conditions of a column temperature of 46 °C and a gradient that varied from isocratic in buffer A from 0 to 10 min, then changed isocratically to 100% buffer B at 30 min, followed by an isocratic segment from 30 to 90 min, were chosen as optimal for resolving the 17 amino acids.

An unexpected result of chromatographing DTDPA-containing samples was a loss of resolving capacity across the entire chromatogram. It proved possible to regenerate the column by passing through it overnight substantial amounts of a cleaning solution composed of 0.05 M sodium citrate, 0.05 M NaCl, and 0.1 M NaOH at 60 °C. The apparent affinity of DTDPA or its reaction product to the polystyrene/divinylbenzenesulfonic acid analytical column suggested that the interfering compound might be removed by passing hydrolysates through a similar resin under conditions at which the amino acids should not bind—high ion strength and high pH. This possibility was investigated with hydrolysates of lysozyme produced in the presence and absence of DTDPA. Lysozyme was chosen to test the effectiveness of the cleanup procedure due to its relatively high cyst(e)ine content. DTDPA-free hydrolysates were chromatographed with or without first passing them through the cleanup column, while DTDPA-containing hydrolysates were chromatographed after passing through the cleanup column. The results in Table 2 show that it is possible to quantitatively recover all of the amino acids from hydrolysates of lysozyme that had been passed through the cleanup column. To quantitatively elute arginine (the most basic amino acid) from the cleanup column, a bed volume of 3 mL and 30 mL of pH 7.5 Na buffer were used. Amino acid profiles of the three different treatments of lysozyme hydrolysates were compared with literature values obtained from sequence data (Canfield and Liu, 1965), and ratios of experimental to literature value were calculated. All ratios are near 1 except for valine and isoleucine, which are known to be released slowly during acid hydrolysis, and methionine, the enhanced recovery of which we assume to be due to protection by the sacrificial oxidation of excess DTDPA. Table 2 also shows that hydrolyzing a protein

Table 4. Amino Acid Profiles (Grams per 100 g of Sample) of Casein Diet and Digesta as a Function of Hydrolysis Time[a]

	casein diet, hydrolysis time of				casein digesta, hydrolysis time of			
amino acid	50 min	75 min	240 min	540 min	50 min	75 min	240 min	540 min
Asp	0.637	0.607	0.793	0.707	1.096	1.174	1.300	1.368
Thr	0.269	0.242	0.398	0.403	0.829	0.926	1.013	1.178
Ser	0.425	0.425	0.629	0.545	0.798	0.850	0.933	0.854
Glu	1.743	1.625	2.211	2.093	1.354	1.446	1.569	1.723
Pro	0.779	0.794	1.090	0.992	1.629	1.747	1.853	1.806
Gly	0.187	0.170	0.198	0.196	2.207	2.243	2.319	2.575
Ala	0.249	0.239	0.313	0.285	0.677	0.734	0.809	0.798
Val	0.251	0.288	0.579	0.585	0.368	0.461	0.661	0.694
Cys-X	0.118	0.106	0.044	0.031	0.330	0.346	0.448	0.338
Met	0.227	0.232	0.258	0.270	0.379	0.408	0.291	0.295
Ile	0.241	0.245	0.463	0.489	0.880	0.939	0.890	0.807
Leu	0.708	0.685	0.931	0.908	0.898	0.964	0.999	1.005
Tyr	0.392	0.369	0.516	0.506	0.342	0.394	0.402	0.438
Phe	0.373	0.355	0.513	0.478	0.345	0.404	0.441	0.472
Lys	0.506	0.512	0.738	0.723	0.452	0.496	0.567	0.590
His	0.166	0.156	0.258	0.249	0.192	0.208	0.226	0.264
Arg	0.199	0.179	0.316	0.322	0.379	0.361	0.475	0.527

[a] Mean values from hydrolysis and chromatography of duplicate samples.

Table 5. Total Recovery (Grams per 100 g of Protein) of Amino Acid Nitrogen as a Function of Hydrolysis Time[a]

	hydrolysis time			
sample	50 min	75 min	240 min	540 min
sorghum diet	82.6	94.9	96.0	99.7
sorghum digesta	69.8	80.4	81.0	76.0
casein diet	78.9	76.3	108.2	103.3
casein digesta	70.4	75.5	81.4	84.2

[a] Mean values from hydrolysis and chromatography of duplicate samples.

in the presence of DTDPA under the conditions employed produces very similar results to standard hydrolysis for all acid-stable amino acids. Barkholt and Jensen (1989) did not report a loss of column resolution as a result of chromatographing DTDPA-containing samples on an ion exchange column. Although a similar chromatographic setup was used, their B buffer was a pH 10 borate buffer, while ours was a pH 7.5 citrate buffer with relatively high ionic strength. They also regenerated their column every 40 runs with a solution of 0.2 N NaOH/7 mM EDTA. Frequent exposure of the column to high pH doubtless prevented the accumulation of the interfering species.

For the DTDPA method to be useful in food science and nutrition research, it must be applicable to mixtures of proteins with carbohydrate and other materials commonly found in foods, feeds, and other biological materials. This applicability was tested by applying the method to diet and digesta samples from a nutritional study in swine (Tuan, 1995).

The effects of the method and hydrolysis time on amino acid profiles from sorghum diet and corresponding digesta and from casein diet and digesta are shown in Tables 3 and 4, respectively. Total recovery of amino acid nitrogen as a function of hydrolysis time are shown in Table 5. Total and individual amino acid recovery was found to depend on both sample and hydrolysis time. The change in amino acid concentration varied with hydrolysis time in a complex manner and differed between diet and digesta samples. Total amino acid recovery (g/100 g of N × 6.25) was higher from diets than from digesta, possibly because of high levels of nonprotein/non-amino acid nitrogen in ileal contents. The

Table 6. Factors for Correcting Amino Acid Content to the Maximum Value

	sorghum diet, hydrolysis time of				sorghum digesta, hydrolysis time of				casein diet, hydrolysis time of				casein digesta, hydrolysis time of			
amino acid[a]	50 min	75 min	250 min	540 min	50 min	75 min	250 min	540 min	50 min	75 min	250 min	540 min	50 min	75 min	250 min	540 min
Asp	1.11				1.13			1.11	1.24	1.31		1.12	1.25	1.17		
Thr*	1.56	1.10			1.24				1.50	1.67			1.42	1.27	1.16	
Ser					1.11		1.12	1.16	1.48	1.48		1.15	1.17			
Glu	1.11				1.13				1.27	1.36			1.27	1.19		
Pro	1.23				1.19				1.40	1.37		1.10	1.14			
Gly			1.16							1.16			1.17	1.15	1.11	
Ala					1.13		1.12	1.17	1.26	1.31		1.10	1.20	1.10		
Val*	2.28	1.50	1.18		1.67	1.25			2.33	2.03			1.89	1.51		
Cys-X*	1.25		1.54	1.17	1.12			1.15		1.11	2.68	3.81	1.36	1.30		1.33
Met*	1.77		1.22				1.20	1.30	1.19	1.16					1.40	1.38
Ile*	1.97	1.44	1.12		1.23				2.03	2.00						1.17
Leu*	1.22				1.15				1.31	1.36			1.12			
Tyr*					1.17			1.11	1.32	1.40			1.21	1.11		
Phe*	1.29	1.14	1.10		1.16				1.38	1.45			1.37	1.17		
Lys*	1.44	1.30	1.17		1.32				1.46	1.44			1.31	1.19		
His*	1.39	1.17			1.44	1.11			1.55	1.65			1.38	1.27	1.17	
Arg	1.37	1.11			1.70		1.21	1.02	1.62	1.80			1.39	1.46	1.11	
no. of factors	13	7	7	1	15	2	4	6	15	17	1	6	15	11	5	3
sum of factors	18.98	8.75	8.48	1.17	18.88	2.35	4.65	8.01	22.34	25.06	2.68	8.28	19.65	14.89	5.95	3.88
sum of factors*	14.17	7.64	7.32	1.17	11.48	2.35	1.20	3.56	14.07	15.27	2.68	381	11.06	8.82	3.73	3.88

[a] Asterisks denote essential and sparing amino acids.

shortest hydrolysis time failed to completely release most amino acids from diets, and even labile amino acids such as threonine increased or leveled off at longer hydrolysis times. As expected, the branched chain amino acids, valine and isoleucine, were released slowly, although the latter appeared to decrease slightly during long-term hydrolysis of digesta samples.

Values of amino acid yield for each sample and hydrolysis time were used to calculate correction factors—the ratio of the maximum yield for the respective amino acid to the yield obtained after the various hydrolysis times. Factors are shown when the correction was >10% as shown in Table 6. On the basis of the assumption that the fewer such factors and the smaller they were, the better, the results were further interpreted by calculating the number and sum of correction factors for the different hydrolysis times. While there were differences among the four samples, generally longer hydrolysis times resulted in better recoveries of most amino acids, even those considered to be labile such as threonine. This was reflected by the lower number and sum of coefficients for 250 and 540 min, when all amino acid residues as well as only those that are nutritionally essential were considered. The recovery of Cys-X, and in some cases methionine, were exceptions, usually being better at shorter hydrolysis/reaction times. Rowan et al. (1992) reported that there was a significant curvilinear effect of hydrolysis time on the yields of all amino acids except tyrosine from diet, ileal digesta, and feces samples hydrolyzed at 110 °C for 16, 24, 48, and 72 h. They observed significant hydrolysis time−source interactions for most amino acids, except isoleucine, lysine, serine and tyrosine for which they found the correction factors to be 1.21 for isoleucine, 1.09 for lysine, and 1.04 for serine regardless of the protein sources. In contrast to our observations, they reported generally higher correction factors for digesta than for diet.

While more extensive studies would be needed to ascertain the degree of variability in amino acid recovery as a function of specific sample, it seems the most careful approach remains to hydrolyze each sample at a range of times and correct the results when time and resources permit. Despite the inherent variability in amino acid recovery, this study has demonstrated that the Barkholt and Jensen (1989) method may be successfully applied to complex food, feed, and digesta samples, allowing the recovery of all protein amino acids except tryptophan from a single hydrolysate.

ABBREVIATIONS USED

Cys-X, reaction product of cystine or cysteine and DTDPA, thought to be the mixed disulfide; DTDPA, 3,3′-dithiodipropionic acid.

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