Pyrolysis of Soybean Protein and an Amino Acid Mixture Having the Same Amino Acid Composition

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Soybean protein and a mixture of amino acids corresponding to the composition of soybean protein were pyrolyzed at 850° in a nitrogen atmosphere. The noncondensable gaseous products and 24 components of the neutral fraction of the pyrolysate have been identified. In addition the relative concentrations of the individual components of the neutral fraction were determined. Representative constituents include naphthalene, benzonitrile, indole, a phenanthrene–anthracene mixture, indene, acenaphthylene, 1- and 2-naphthonitriles, and pyrene. The same neutral compounds were formed from the pyrolysis of the soybean protein and from the amino acid mixture. For most components, the relative amounts formed in each pyrolysis were approximately the same. Benzonitrile and the naphthonitriles were formed in larger amounts from the protein than from the amino acid mixture. The results indicate that data obtained from pyrolysis of amino acid mixtures are useful in predicting the composition of protein pyrolysates.

In previous work (Patterson *et al.*, 1969, 1971, 1973) we have pyrolyzed several common amino acids in a nitrogen atmosphere at 850°.

Since amino acids occur in nature primarily in the form of proteins, the present investigations have been performed to determine if the pyrolysis of a mixture of amino acids yields the same results as the amino acids pyrolyzed individually. In previous work, Patterson *et al.* pyrolyzed an equimolar mixture of phenylalanine and tryptophan in a nitrogen atmosphere at 850° and the compounds obtained were accounted for by comparison to the products of the individually pyrolyzed amino acids. There was observed, however, a large decrease in the production of aromatic hydrocarbons in the mixture. The presence of a second amino acid was found to influence the yields of certain components in the pyrolysate. For example, the fluoranthene–pyrene–benzofluorene–chrysene mixture and phenanthrene–anthracene mixture yields were considerably decreased, while the naphthalene and indole yields were increased.

In a recent report, Higman *et al.* (1970) compared pyrolysate compositions obtained from the pyrolysis of casein and collagen at 840° in nitrogen with those obtained from glycine and proline under like conditions. The product mixtures thus obtained were exceedingly complex, but several of the major components were characterized.

In the present investigation, a protein of known amino acid composition was pyrolyzed. A homogeneous mixture of amino acids, having the same relative concentrations as present in the protein, was pyrolyzed under identical conditions. In this way the high-temperature chemical transformation of a complex mixture of amino acids was compared to the high-temperature chemical transformation of the same amino acids joined *via* amide linkages.

MATERIALS AND METHODS

Ultraviolet spectra were measured in cyclohexane using a Perkin-Elmer Model 202 spectrophotometer, and infrared spectra were measured in chloroform or carbon tetrachloride using a Beckman IR-8 spectrophotometer equipped with a mirror beam condenser. Glpc (gas–liquid phase chromatography) retention times were measured and separation of the pyrolysate constituents was carried out on an F&M Model 810 gas chromatograph.

Materials. The soybean protein pyrolyzed in this study was Alpha Protein (commercial name for highly purified soybean protein) obtained from the Nutritional Biochemical Corporation. The protein was dried to constant weight prior to pyrolysis for approximately 17 hr at 80–85°. A weight loss of 8.2% was observed during drying.

The amino acid mixture was prepared from commercially available samples (Nutritional Biochemical Corporation) so as to have the composition described in Table I. The substances were carefully mixed so as to produce a homogeneous sample. Lysine was used in the form of the monohydrochloride, and the calculated weight of lysine in the mixture included the hydrogen chloride. Therefore the weight of free lysine is approximately 20% less than in the protein.

Pyrolyses. The pyrolyses were carried out in the apparatus previously described (Patterson *et al.*, 1969, 1971, 1973) using 30 ml of Berl saddles and a nitrogen flow of 60 ml/min. The protein or the amino acid mixture was added to the pyrolysis tube at the same constant rate by means of a rotating screwfeed driven by a Troemer monodrum unit. Sample addition was discontinued at the appearance of pressure build-up in the apparatus arising from clogging of the pyrolysis tube in the region containing the Berl saddles.

The liquid products were collected in two traps, each of which was cooled in a Dry Ice–chloroform–carbon tetrachloride mixture. Gases which were not condensed by these traps were examined by infrared spectroscopy using a 100-mm gas cell. Identifications were based upon comparisons of the absorption bands observed with those reported in the literature (Pierson *et al.*, 1956) and with those obtained from authentic samples.

The pyrolysate was heated in a water bath at 100° (the gases evolved were analyzed by infrared spectroscopy) and the distillate collected. The residue was extracted with ether and the ether-soluble material separated into acidic, neutral, and basic fractions by extraction with successive portions of 5% HCl and 5% NaOH, each saturated with NaCl. The resulting ether layer containing the neutral fraction was washed until neutral with H_2O saturated with NaCl and then dried over Na_2SO_4 overnight. After evaporation of the ether, the weight of the neutral fraction was determined.

Separation and Identification of Components. Components of the neutral fraction were separated by glpc using a 25 ft × 0.375 in. 20% Apiezon L column (Anakrom 50/60 U) heated at 100° for 8 min and then programmed at 4°/min to 270°. The final temperature was maintained for an extended period to ensure elution of high boiling components. A second column was employed in an attempt to achieve a better separation and to provide confirmatory evidence of the results obtained from the Apiezon L column. This was a 22 ft × 0.375 in. 25% UCW-98

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Table I. Composition of Soybean Protein[a]

Amino acid	Wt % AA/ total protein	Amino acid	Wt % AA/ total protein
Arginine	7.0	Threonine	3.9
Histidine	2.5	Leucine	7.6
Lysine	6.6	Isoleucine	5.8
Tyrosine	3.2	Valine	5.2
Tryptophan	1.2	Glutamic acid	18.5
Phenylalanine	4.8	Aspartic acid	8.3
Cystine	1.2	Glycine	3.8
Methionine	1.1	Alanine	4.5
Serine	5.6	Proline	5.4

[a] R. J. Block and R. W. Weiss, "Amino Acid Handbook: Methods and Results of Protein Analysis," C. C. Thomas, Springfield, Ill., 1956, p 345.

column (Anakrom 50/60 U) heated isothermally at 90° for 4 min and then programmed at 2°/min to 235°. The above two columns were used to characterize those components of the neutral fraction with retention times less than and including carbazole and phenanthrene–anthracene.

Components of the neutral fraction with retention times greater than the phenanthrene–anthracene mixture and carbazole were separated on a 12 ft × 0.375 in. 10% SE-30 column (Anakrom 50/60 U), heated isothermally at 100° for 4 min, and then programmed at 2°/min to 275°. The final temperature was maintained for 100 min to ensure elution of all high boiling material.

Identifications of components are based on comparisons of glpc retention times, ultraviolet spectra, and infrared spectra with those obtained from authentic samples. Since it was difficult to obtain consistent absolute retention times, the following procedure using relative retention times was adopted. Thus, for the Apiezon L and UCW-98 columns, those compounds with retention times less than 2-methylnaphthalene were identified by comparison of authentic sample retention times relative to the retention time of benzonitrile. Those compounds appearing from 2-methylnaphthalene up to and including fluorene were identified by comparison of authentic sample retention times relative to the retention time of naphthalene. Those compounds appearing after fluorene were identified by comparison of authentic sample retention times relative to the retention time of fluorene. For the SE-30 column, all compounds were identified by comparison of authentic sample retention times relative to the retention time of phenanthrene.

Estimations of relative abundances of constituents are based on area per cent values obtained from glpc using the height times the width at half-height method. The results are reported in the tables.

RESULTS AND DISCUSSION

Soybean protein was chosen as the protein to be pyrolyzed, because its amino acid composition (Table I) is firmly established and it is readily available. Both the soybean protein and the corresponding amino acid mixture were pyrolyzed at 850° under a nitrogen atmosphere using identical reaction and work-up conditions. The following noncondensable gases were identified in the pyrolysates of the protein and the amino acid mixture: carbon dioxide, carbon monoxide, carbon oxysulfide, carbon disulfide, methane, ethylene, acetylene, ammonia, and hydrogen cyanide. Data for typical pyrolyses at 850° are summarized in Table II. As shown in Table II, the pyrolysis of soybean protein yields a larger neutral fraction and twice as much residue per gram pyrolyzed as the pyrolysis of the amino acid mixture.

Analyses of the neutral fractions were carried out by glpc using Apiezon L and UCW-98 columns for components with retention times less than and including carbazole and the phenanthrene–anthracene mixture and an SE-30 column for those components with retention times which were greater. The UCW-98 column was effective in separating components which appeared as mixtures when the Apiezon L column was used. The following groups of substances are representative of the mixtures separated: indene and *m*- and *p*-toluonitrile, indole and cinnamonitrile, and 1- and 2-naphthonitrile and acenaphthylene.

Table II. Weights of Products from Pyrolysis of Soybean Protein and Amino Acid Mixture at 850°

Substance pyrolyzed	Wt pyrolyzed, g	Wt of residue, g	Wt of pyrolysate, g	Wt of neutrals, g
Protein	27.14	5.73	12.48	1.39
Amino acid mixture	29.21	2.29	13.08	1.02

The areas of the peaks appearing on the SE-30 chromatogram were coordinated with the areas of the peaks appearing on the UCW-98 chromatogram by multiplying the component areas obtained from the SE-30 chromatogram by the ratio of areas of carbazole from the UCW-98 and SE-30 chromatograms. The peak areas from the UCW-98 chromatogram and the coordinated peak areas from the SE-30 column were summed, and a percentage distribution of the components of the neutral fraction was obtained.

The components identified in the neutral fraction and their relative concentrations are reported in Table III in order of their appearance on the UCW-98 and SE-30 columns. In previous studies on the pyrolysis products of amino acids (Patterson *et al.*, 1969, 1971, 1973), the relative concentrations of constituents were reported as grams produced per mole of substance pyrolyzed. So that comparisons could be made between this study and earlier work, constituent concentrations are also reported as grams per mole pyrolyzed. For this purpose, 1 mol of amino acid mixture was defined as 130.0 g and was calculated as follows. In 100.0 g of amino acid mixture there is 0.7641 mol of amino acid present. This was computed from the weight of each amino acid present in 100.0 g of mixture (*cf.* Table I) and the particular amino acid's molecular weight. Dividing 100.0 g by 0.7641 mol yields a theoretical mol wt of 130.9 g/mol. Thus, in 130.9 g of amino acid mixture, there is 1.00 mol of amino acid present. The weights of constituents from the neutral fraction of the amino acid mixture pyrolysis are presented in grams per theoretical mole pyrolyzed.

The weights of constituents from the neutral fraction of the protein pyrolysis were represented in a way permitting comparison with the results of the amino acid mixture pyrolysis. Since reporting the weights of constituents from the neutral fraction of the protein pyrolysis in grams per 130.9 g pyrolyzed would not take into account the fact that amino acids, existing in the free state, contain an additional molecule of water which is not present when the amino acids were combined *via* amide linkages of protein, the weight of 1 mol of water was subtracted from the weight of a theoretical mole of amino acid. The corrected theoretical mole of protein weighs 112.9 g. (In 112.9 g of protein, there is 1.000 mol of amino acid residues, *i.e.*, amino acids less 1 mol of water. The N-terminal amino acid and C-terminal amino acid in the protein chain cumulatively contain a molecule of water which is a part of the protein. The mol wt for soybean protein is 361,800. Dividing this by the corrected theoretical mol wt (112.9 g/mol) yields the approximate number of amino acids joined together in the protein (3200). Neglecting the terminal water molecular results in a negligible error of approximately 1 in 3200 or 0.03%.) The weights of constitu-

Table III. Neutral Pyrolysate Components from the Pyrolysis of Amino Acid Mixture and Soybean Protein

Component	Amino acid mixture		Protein	
Total neutrals, g	4.55[a]		5.78[b]	
Phenylacetylene[c]	0.003	0.07[e]	0.004	0.06[e]
Styrene[c]	0.04	1.0	0.05	0.8
Benzonitrile	0.65	14	1.2	21
Indene	0.20	4.4	0.16	2.7
o-Toluonitrile	0.09	2.0	0.10	1.8
m-Toluonitrile	0.05	1.0	0.09	1.5
p-Toluonitrile	0.02	0.5	0.05	0.9
Naphthalene	1.1	24	1.1	19
Cinnamonitrile	0.05	1.1	0.21	3.6
Indole	0.33	7.2	0.47	8.2
2-Methylnaphthalene	0.05	1.0	0.04	0.7
1-Methylnaphthalene	0.04	0.9	0.04	0.6
Biphenyl	0.09	2.0	0.09	1.6
2-Vinylnaphthalene	0.06	1.2	0.04	0.7
Acenaphthalene	0.20	4.4	0.16	2.7
1-Naphthonitrile	0.20	4.3	0.30	5.1
2-Naphthonitrile	0.18	3.9	0.28	4.9
Fluorene	0.07	1.6	0.06	1.1
Phenanthrene–anthracene	0.26	5.7	0.22	3.7
Carbazole	0.08	1.8	0.07	1.3
Fluoranthene	0.05	1.1	0.04	0.6
Pyrene	0.12	2.6	0.11	1.8
Chrysene mixture[d]	0.06	1.2	0.06	0.9
Unidentified components		13		15
		100%		100%

[a] In grams per theoretical mole (130.9 g) pyrolyzed. [b] In grams per corrected theoretical mole (112.9 g) pyrolyzed. [c] Present also in 100° distillate. [d] Mixture of chrysene, triphenylene, and 1,2-benzanthracene. [e] Area per cent as determined by glpc.

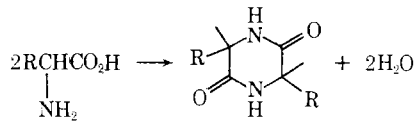
ents from the neutral fraction of the protein pyrolysis, appearing in Table III, are represented in grams per corrected theoretical mole pyrolyzed.

An indication of the reproducibility of the pyrolysis experiments was obtained from a calculation of the relative standard deviation of the mean pyrolysate weight (amino acid mixture, 4%; protein, 2%) and the mean neutral fraction weight (amino acid mixture, 5%; protein, 6%) based on four pyrolysis experiments.

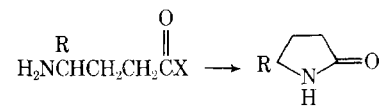
The results of these pyrolyses show that a larger neutral fraction is obtained from the pyrolysis of protein than from the pyrolysis of the amino acid mixture. While the pyrolysis of both protein and amino acid mixture produces approximately the same quantities of hydrocarbons, the data indicate that aromatic nitriles (benzonitrile, toluonitriles, naphthonitriles, cinnamonitrile) and indole are produced in larger quantities in the protein pyrolysis.

The formation of cyano compounds may be postulated as resulting from the combination of a cyano radical and a hydrocarbon radical or the combination of hydrogen cyanide and a benzyne-type material. Thus, since the protein pyrolysis produces more cyano compounds, it seems that there is a greater tendency to form cyano radicals or hydrogen cyanide in the pyrolysis of a protein than in the pyrolysis of an amino acid mixture.

Johnson and Kang (1971) after carrying out a systematic study of hydrogen cyanide formation from the pyrolysis of amino acids and related compounds proposed a cyclization mechanism for the formation of hydrogen cyanide from the pyrolysis of amino acids in which two amino acids combine to form the piperazinedione. Primary cleavage would yield the imine RCH=NH, which in turn undergoes dehydrogenation to form the alkyl cyanide or dialkylation to form hydrogen cyanide.

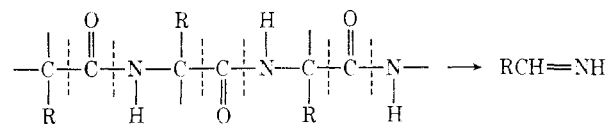


In the Johnson and Kang pyrolysis of isomeric aminobutyric acids, aminodicarboxylic acids, and aminodicarboxylic acid derivatives, unusually high yields of hydrogen cyanide were obtained in those cases in which suitable intramolecular cyclization was possible, *i.e.*



where R = H and COOH and X = OH and NH_2. It was demonstrated that such cyclization by intramolecular reaction generated the necessary intermediate easier than a bimolecular reaction. In cases in which intramolecular cyclization could lead to the more sterically strained four-membered rings, only slightly higher yields of hydrogen cyanide were formed.

In the pyrolysis of the protein, the data suggest that cyano species form more readily than in the pyrolysis of the amino acid mixture. This could be due to the fact that the amino acids have to generate a cyclic intermediate, *via* amide linkage, before forming a cyano compound or radical. This intermediate could be formed by means of an intermolecular or intramolecular process. Proteins, however, existing already in an amide linked system, could react more directly to form the imine (precursor to cyano radicals and compounds).



One of the pathways by which protein undergoes pyrolysis may well be *via* the piperazinediones. The thermal degradation of peptides has been reported to give piperazinediones (Lichtenstein, 1938; Poroshin, 1959), and the yields of certain piperazinediones from actinomycin D (at 400°) are as high as 59% (Mauger, 1971). The necessity of forming an amide-linked intermediate from the free amino acid might allow competing reactions (*e.g.*, deamination) to proceed to a larger extent in the amino acid mixture pyrolysis than in the protein pyrolysis. In the Johnson and Kang study, it was found that the pyrolysis

Table IV. Quantitative Comparison of High Molecular Weight Compounds from Selected Pyrolyses (Gram/Mole Pyrolyzed)

Pyrolysis	Phenan.–anthr.	Fluor-anthene	Py-rene	Chrysene (mixt.)
Leucine	0.25	0.09	0.11	0.05
Amino acid mixt.	0.26	0.05	0.12	0.06
Protein	0.22	0.04	0.11	0.06
Phe–Trp mixt.	0.5	0.06	0.11	0.06

of 2,5-piperazinedione, the proposed cyclic intermediate in the glycine pyrogenesis of hydrogen cyanide, produced over twice as much hydrogen cyanide (per molecule of N orginally present) as the pyrolysis of glycine itself.

A comparison was made between the amounts of the identified high molecular weight compounds obtained from the protein and amino acid mixture pyrolyses and previously published amounts of the same compounds from the pyrolysis of individual amino acids (*cf.* Patterson *et al.*, 1969, 1971; Smith *et al.*, 1973). The results of the protein pyrolysis and amino acid mixture, in respect to high molecular weight compounds, were very similar to those of leucine (Table IV). This is significant because almost all of the individually pyrolyzed amino acids gave much higher amounts of these substances per mole pyrolyzed than did leucine. If, in the case of high molecular weight compounds, the pyrolysis of a mixture of amino acids reflected an average of individually pyrolyzed amino acids, the amounts of high molecular weight compounds would have been higher.

Comparison was also made between the identified high molecular weight compounds from the protein and amino acid mixture pyrolyses and the published amounts of the same compounds from the pyrolysis of an equimolar mixture of phenylalanine and tryptophan (Table IV). The pyrolysis of the phenylalanine–tryptophan mixture represented the pyrolysis of a mixture of only aromatic amino acids. Yet, it is interesting to note that the phenylalanine–tryptophan results are similar to the protein and amino acid mixture results, in respect to the high molecular weight compounds.

Our pyrolytic studies at 850° show that pyrolyses of amino acids give the same results qualitatively and, with a few exceptions, quantitatively, as those obtained by pyrolyses of protein having the equivalent amino acid composition. These results give added significance to our previous studies on pyrolysis of individual amino acids and of simple mixtures and should be useful in the future in predicting pyrolytic products of other proteins.

LITERATURE CITED

Higman, E. B., Schmeltz, I., Schlotzhauer, W. S., *J. Agr. Food Chem.* **18**, 636 (1970).
Johnson, W. R., Kang, J. C., *J. Org. Chem.* **36**, 189 (1971).
Lichtenstein, N., *J. Amer. Chem. Soc.* **60**, 560 (1938).
Mauger, A. B., *Chem. Commun.*, 39 (1971).
Patterson, J. M., Baedecker, M. L., Musik, R., Smith, W. T., Jr., *Tob. Sci.* **13**, 26 (1969).
Patterson, J. M., Chen, W., Smith, W. T., Jr., *Tob. Sci.* **15**, 98 (1971).
Patterson, J. M., Haidar, N. F., Papadopoulos, E. P., Smith, W. T., Jr., *J. Org. Chem.* **38**, 663 (1973).
Pierson, R. H., Fletcher, A. N., Gantz, E. S. C., *Anal. Chem.* **28**, 1218 (1956).
Poroshin, K. T., *Bull. Acad. Sci. USSR*, 2055 (1959).
Smith, W. T., Jr., Patterson, J. M., Haidar, N. F., unpublished results, 1973.

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Glucosinolate Determination in Cruciferous Seeds and Meals by Measurement of Enzymatically Released Glucose

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Hydrolysis of glucosinolates by thioglucoside glucohydrolase EC 3.2.3.1 (thioglucosidase) releases β-D-glucose. The glucose was measured colorimetrically after specific enzymatic oxidations with readily available reagents containing a glucose oxidase, peroxidase, and chromogen. By this procedure glucosinolates were determined successfully in seed extracts after interfering substances were removed with charcoal. Samples containing glucosinolates equivalent to 0.01–0.15 mg of glucose were analyzed with either added or endogenous thioglucosidase. In the procedure involving addition of thioglucosidase, endogenous enzyme was first heat-inactivated and free glucose was determined. This modification has been used in assessing changes during seed storage and processing. Crambe seed analyses by the two methods agreed and were within 10% of results by an established independent method. Relative standard deviation by three tests was 3.3, 4.2, and 6.5%. Test paper impregnated with the enzymes and chromogen permitted screening of plant breeding samples for glucosinolate content.

The plant family Cruciferae includes rape (*Brassica campestris, B. napus*), mustard (*B. juncea, B. hirta*), and crambe (*Crambe abyssinica*). Seeds of all members of the family so far examined contain glucosinolates (thioglucosides) which are the source of organic compounds that contribute to the flavor of these plants but which also may be harmful when the defatted seed meal is consumed in large amounts by livestock. Reviews are available on the chemistry of these compounds (Ettlinger and Kjaer, 1968), problems related to the use of the plants in food or feed (VanEtten and Wolff, 1973), and removal of glucosinolates by processing or by plant breeding (Tallent, 1972).

Analytical methods include estimating the split products, such as mustard oils and goitrin, formed from each glucosinolate under specific conditions of hydrolysis by

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