Enzymatic Hydrolysis of Food Protein for Amino Acid Analysis. I. Solubilization of the Protein

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Treatment with 8 M urea and papain (1 mg/10 mg of food protein) for 24 h at 37 °C rendered food proteins from mixed 1-day food samples completely soluble. Separation of urea from the protein hydrolysate was obtained on a highly acidic cation exchanger. After these procedures the food proteins were obtained in a completely water soluble, partially hydrolyzed form suitable for further treatment with insolubilized peptidases to obtain a complete enzymatic hydrolysis for amino acid analysis.

The nutritive value of food proteins depends on their content of essential amino acids in combination with the digestibility of the proteins. The amino acid analysis of protein is therefore an essential part of their nutritional evaluation.

Some amino acids can be determined by specific reactions while they are still attached to the protein molecule. These methods are limited to a few amino acids, such as tryptophan, tyrosine, cystine, and cysteine. A complete assessment of the amino acid pattern requires hydrolysis of the peptide linkages before separation by ion-exchange chromatography (Moore and Stein, 1951) or by gas chromatography (Gehrke et al., 1971).

Methods for hydrolysis available today indicate treatment with acid or alkali and give many side reactions influencing the recovery of amino acids.

The most commonly used acidic reagent is 6 N HCl, which can be readily removed from the hydrolysate by evaporation under a vacuum. Acid hydrolysis causes partial destruction of cysteine and tyrosine, and more or less complete destruction of tryptophan. Incomplete liberation of amino acids such as valine and isoleucine and racemization and destruction of certain amino acids such as serine and threonine cause the amino acid composition of the acid hydrolysate to differ from that of the protein from which it is derived. This effect is more pronounced in the presence of other compounds, especially carbohydrates. Futhermore, after acid hydrolysis glutamine and asparagine are recovered as their respective acids. Lysine bound in the Maillard complex and peptides containing D-amino acids, normally not available to the enzymes of the intestinal tract, will also be hydrolyzed (Finot, 1973).

Tryptophan can be recovered after alkaline hydrolysis, and the sulfur-containing amino acids can be assayed after oxidation with performic acid followed by acid hydrolysis. Such methods make it possible to overcome some of the disadvantages in acid hydrolysis, but a large number of relatively laborious procedures have to be used for a complete assay of the amino acid pattern (Roach and Gehrke, 1970). Enzymatic hydrolysis of the protein would be a milder method less likely to destroy the amino acids.

Hill and Schmidt (1962) succeeded in hydrolyzing peptides and proteins oxidized with performic acid completely with papain followed by leucine aminopeptidase and prolidase. There are certain drawbacks associated with this procedure. The added enzymes will contaminate the sample and cannot be reused. Furthur, the enzyme preparations, especially prolidase and aminopeptidase, might not remain stable under prolonged periods of storage. However, it is possible to attach enzymes to insoluble carriers such as CMC and Sepharose with retained activity and enhanced stability (Mosbach, 1971). The immobilized enzymes can be used in excess, are readily separated from the incubation mixture, and can then be used again.

Bennet et al. (1972) have used trypsin, chymotrypsin, aminopeptidase M, and prolidase covalently bound to CNBr-Sepharose to hydrolyze a number of peptides and S-aminoethylated proteins. Similar investigations were also conducted by Chin and Wold (1974) and by Royer and Andrews (1973).

In these investigations the samples analyzed have consisted of soluble proteins. The method cannot be applied to food proteins without modifications, since most of the food proteins are insoluble and thus unavailable to the enzymes when these are attached to insoluble carriers. We have therefore studied the possibilities of solubilizing the proteins in the food samples in order to convert them into a form that can be attacked by the insolubilized proteases. In the present investigation, solubilization by mild acid hydrolysis and by treatment with urea alone and in combination with papain was studied.

MATERIALS AND METHODS

Food Samples. Fat-extracted and lyophilized 1-day specimens of mixed food from a duplicate-portion study (Borgström et al., 1975) were used.

Papain. Papain (2× crystallized in a sodium acetate suspension) was purchased from Sigma Chemical Co., St. Louis, Mo. The activity measurements were performed with pH stat equipment. 2-*N*-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) (Sigma Chemical Co.) was used as the substrate. The initial concentration was 50 mM. The incubation medium contained thioglycollic acid, 5 mM, and EDTA, 2 mM, as the activating agents, and the reaction was performed under a nitrogen atmosphere at pH 7.6. The enzyme preparations used had a specific activity of 10–15 measured as micromoles per liter of hydrolyzed BAEE per minute per milligram of enzyme and calculated from the initial reation rate. When the activity of papain in the urea solution was determined, urea was also present in the enzyme assay mixture.

Mild Acid Hydrolysis. The samples were heated in sealed tubes along with the appropriate quantity of hydrochloric acid in a water bath or hot-air oven. At the end of the hydrolysis period, the contents were centrifuged to collect the insoluble protein. The nitrogen content of this fraction was determined. Time, temperature, and acid concentration for the mild acid hydrolysis are as in Figure 1.

Amino Acid Analysis. After acid hydrolysis with 6 N HCl at 110 °C for 20 h, a gas chromatographic assay was

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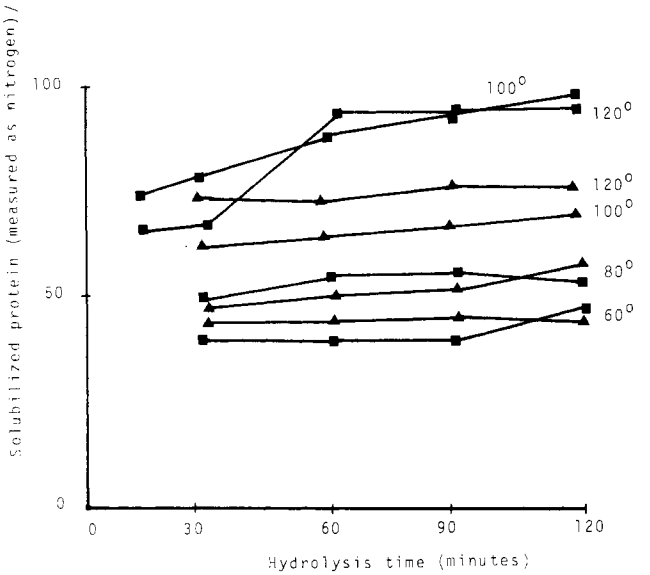

Figure 1. Effect of mild acid treatment on the solubility of proteins in a food sample as a function of temperature and time: (■) 0.1 N HCl; (▲) 0.01 N HCl.

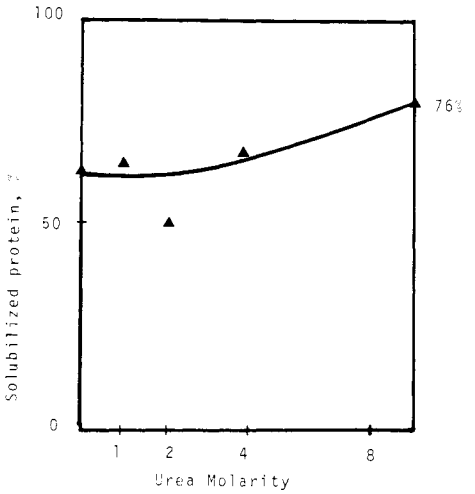

Figure 2. Effect of urea concentration on the solubility of proteins in a food sample.

performed as described by Kaiser et al. (1974). Prior to derivatization to *N*-trifluoroacetyl-*N*-butyl esters, the hydrolysate was cleaned up on a strongly acidic cation exchanger (Amberlite IR-120, BDH).

Nitrogen Assay. The Kjeldahl method was used.

Treatment with Urea and Papain–Urea Solutions. Urea, "aristar" quality, was purchased from BDH, Poole, England. The urea solutions, containing 0.1 M sodium borate and adjusted to pH 7.6, were prepared immediately before use. When papain was present in the solution, this also contained 5 mM thioglycollic acid and 2 mM EDTA.

Five milliliters of the solution was added to a weighed amount of the food sample, containing approximately 10 mg of protein, and the extractions were performed in sealed tubes employing end-over-end rotation at 37 °C. When used, 40 μl of concentrated papain solution (25 mg/ml) was added. After extraction the insoluble residue was separated from the urea solution by centrifugation. The centrifugate was washed with distilled water and analyzed for amino acids as described above. The yield was compared with that from an untreated sample, and the protein solubility could be calculated.

Reagents. The reagents not specified above but used in the experiments were all of analytical grade.

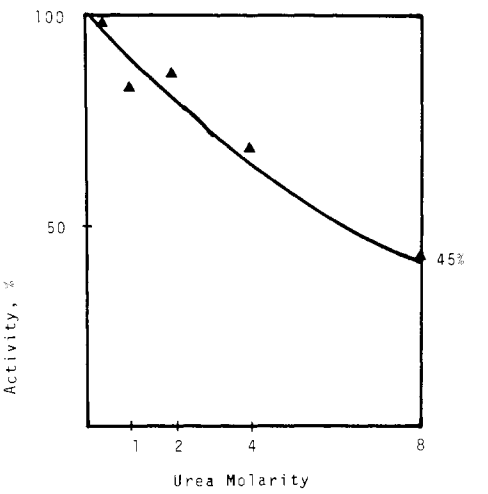

Figure 3. Effect of urea concentration on the activity of papain expressed as percent of the activity in water.

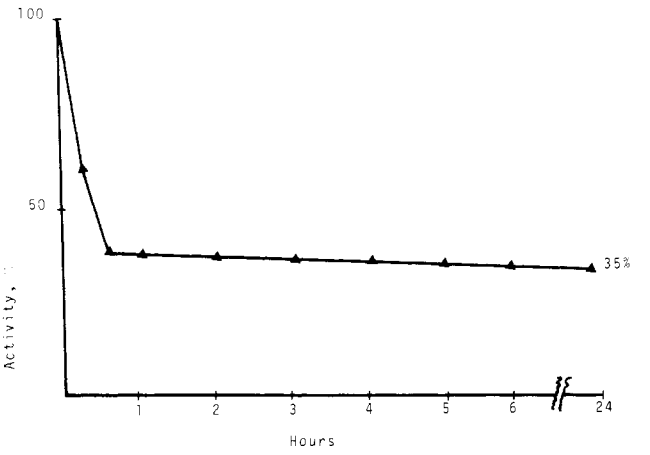

Figure 4. Effect of incubation time on the activity of papain in 8 M urea expressed as percent of the activity in water.

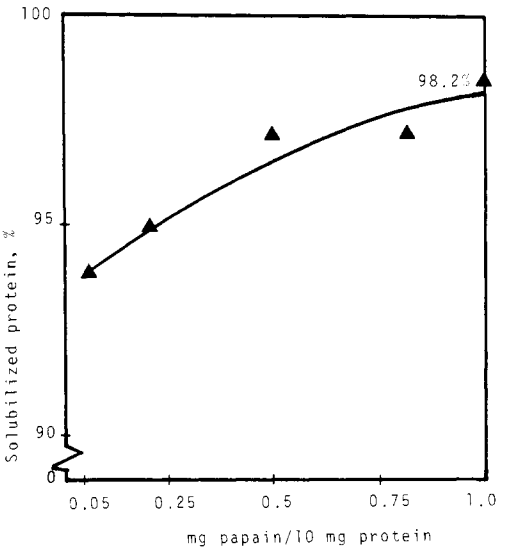

Figure 5. Effect of papain to protein ratio on the solubility of proteins in a food sample in 8 M urea.

RESULTS AND DISCUSSION

Mild Acid Hydrolysis. Treatment with 0.01 and 0.1 N HCl was tried in order to obtain a partial hydrolysis that was sufficient to cause solubilization but mild enough to prevent destruction of acid-labile amino acids. Figure 1

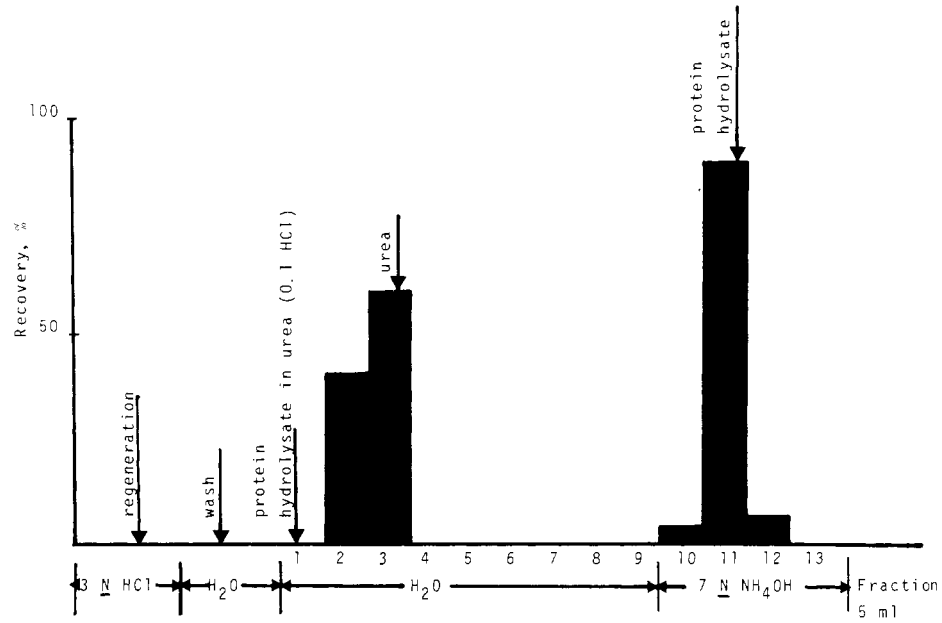

Figure 6. Separation of urea from the protein hydrolysate using a strongly acidic cation exchanger.

Table I. Solubilization of Protein in Ten Different Food Samples by Papain in 8 M Urea

Sample	% solubilized	Sample	% solubilized
12c	97.7	13e	98.7
13a	97.4	13f	99.0
13b	98.3	13g	98.4
13c	98.3	22b	98.0
13d	97.0	22f	98.7

shows the effect of 0.01 and 0.1 N HCl at various temperatures during 30–120 min. At 60–80 °C the temperature was more important for solubilization than the strength of the acid. As the temperature rises the effect of acid strength becomes pronounced. Almost complete solubilization was obtained in 0.1 N HCl at 100–120 °C for 2 h. Such conditions, however, are too drastic to guarantee quantitative recovery of the acid-labile amino acids.

Urea Treatment. Urea was classified by von Hippel and Schleich (1969) as a hydrogen bond former and hydrophobic bond affector, and it competes more efficiently than water for hydrogen-bonding groups. The unfolding of the molecule is further favored by the interaction between urea and the hydrophobic groups, which in addition would make more hydrolytic sites available to enzymes. Figure 2 shows the solubility of food proteins in urea solutions of various concentrations after extraction for 24 h at 37 °C. Increased urea concentration causes increased solubility. In 8 M urea 78% of the protein fraction was soluble.

Papain–Urea Treatment. *Activity and Stability of Papain in Urea.* As shown in Figure 3, the activity of papain, measured with BAEE as the substrate, decreases with increased urea concentration. The activity was measured after exposing the papain to urea in solutions containing activating agents for 1 h. In 8 M urea 45% of the original activity remained. These results are not in agreement with those reported by Nakamura and Soejima (1970) and Hill et al. (1959) using BAEE as the substrate, although enhanced papain activity in 8 M urea was found for the larger substrate casein (Nakamura and Soejima, 1970). Prolonged exposure to 8 M urea at 37 °C caused only a slow decrease in activity in addition to the 45% decrease observed during the first 40-min period. Thus, after 24 h 35% of the original papain activity still remained (Figure 4). This indicates that there was no significant degree of autodigestion nor inhibition due to inactivation of SH groups essential for activity. Thus, it is possible to incubate food proteins with papain in 8 M urea solutions for 24 h.

Solubilization with Urea and Papain. Even small amounts of papain considerably enhanced the solubility of food proteins in 8 M urea after a 24-h incubation at 37 °C. Figure 5 shows the effect of variation of papain to food protein ratio in one sample. An almost complete solubilization was obtained with 1 mg of papain per 10 mg of food protein. Table I shows the solubilization of 10 different food samples with the papain/food protein ratio of 1/10. In all the samples at least 97% of the protein (mean 98.2%) was solubilized.

Separation of Urea from Solubilized Protein. Completion of the protein hydrolysis with other enzymes requires separation of the solubilized and partially hydrolyzed proteins from the urea. For this purpose, the following procedure was elaborated. The pH of the solution was adjusted to 1.0 by addition of 1 N HCl, and then an amount equivalent to 3 mg of protein was applied to a strongly acidic, cation-exchanger column (10 cm × 3 mm with Dowex 50W-X2 (H^+) high porosity). Urea was washed out with deionized water and elution was then carried out with 7 N NH_4OH. The elution pattern is shown in Figure 6. The recovery was calculated by analyzing the amino acid content in each fraction. All the urea was washed out well before the sample was eluted and recovery of the hydrolysate was complete in a couple of fractions. The fractions containing the protein hydrolysate were evaporated. The residue was completely soluble in small volumes of 0.1 N borate buffer (pH 7.6).

Experiments are in progress to hydrolyze this material completely to free amino acids using insolubilized proteases and peptidases.

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LITERATURE CITED

Bennet, H. P. J., Elliot, D. F., Evans, B. E., Lowry, P. J., McMartin, C., *Biochem. J.* **129**, 695 (1972).

Borgström, B., Nordén, Å., Åkesson, B., Jägerstad, M., *Scand. J. Soc. Med.*, Suppl. 10 (1975).

Chin, C. C. Q., Wold, F., *Anal. Biochem.* **61**, 379 (1974).
Finot, P. A., "Proteins in Human Nutrition", Porter, J. W. G., Rolls, B. A., Ed., Academic Press, New York, N.Y., 1973, Chapter 34.
Gehrke, C. W., Zumwalt, R. W., Kuo, K., *J. Agric. Food Chem.* **19**, 605 (1971).
Hill, R. L., Schmidt, W. R., *J. Biol. Chem.* **237**, 389 (1962).
Hill, R. L., Schwartz, H. C., Smith, E. L., *J. Biol. Chem.* **234**, 572 (1959).
Kaiser, F. E., Gehrke, C. W., Zumwalt, R. W., Kuo, K. C., *J. Chromatogr.* **94**, 113 (1974).
Moore, S., Stein, W. H., *J. Biol. Chem.* **192**, 663 (1951).
Mosbach, K., *Sci. Am.* **224**, 26 (1971).
Nakamura, K., Soejima, M., *Agric. Biol. Chem.* **34**, 489 (1970).
Roach, D., Gehrke, C. W., *J. Chromatogr.* **52**, 393 (1970).
Royer, G. P., Andrews, J. P., *J. Biol. Chem.* **248**, 1807 (1973).
von Hippel, P. H., Schleich, R., "Structure and Stability of Biological Macromolecules", Timasheff, S. N., Fasman, G. D., Ed., Marcel Dekker, New York, N.Y., 1969, Chapter 6.

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Application of Chemical and Biological Assay Procedures for Lysine to Fish Meals

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Eight samples of capelin meals differing in freshness of raw material, treatment with formaldehyde, and proportion of solubles were used for this collaborative study in four laboratories. They were subjected to two procedures of bioassay for lysine based on chick growth rates and the results gave similar ranking of the samples, though absolute values differed. The chick results correlated closely with results from a rat assay and with fluorodinitrobenzene (FDNB)-reactive lysine, dye binding with Acid Orange 12, and total lysine values. The potency of different samples as sources of lysine for chicks was mainly determined by their total content of lysine, but the addition of excess levels of formalin to the fish immediately prior to processing reduced potency about 13% and this reduction was not demonstrated by any of the chemical methods used. There were considerable differences between laboratories in the absolute values for FDNB-reactive lysine.

Fish meal usually commands a higher price than other high protein feedstuffs, and a large part of this premium is explained by its amino acid composition being suited to balance the relative deficiencies of cereal proteins for monogastric animals. In particular, the high lysine content of fish protein compensates for the relatively low lysine in cereal proteins.

Carpenter et al. (1957) suggested that the chemical measurement of lysine reactive to fluorodinitrobenzene (FDNB) provided a better, and more convenient, measure of the value of processed animal proteins as a source of lysine than did total lysine as measured by either column chromatography or microbiological assay, after acid hydrolysis. Since then many procedures for measuring reactive lysine (using FDNB or other reagents) have been devised and applied to animal protein feedstuffs (cf. review by Carpenter, 1973).

In many studies a high correlation has been found between the ranking of samples according to FDNB-reactive lysine and according to feeding tests, in particular when the samples had been subjected to extreme temperature treatments. But in some other studies, with commercial fish meals, there was not a good correlation (see Carpenter, 1973). A lack of correlation between FDNB-reactive lysine and feeding tests could be due to the biological assay in question not being sensitive to variations in available lysine perhaps because the first limiting amino acids in the study were methionine and cystine, rather than lysine. Furthermore, it may be that while some reactions (e.g., those occurring at high temperatures) affect FDNB reactivity and biological availability to the same extent, other reactions do not. Finally, a lack of correlation between FDNB–lysine and feeding tests may be due to unrecognized sources of error in the use of either procedure.

These questions have an immediate practical interest as regards the methods to be used for quality control by both processors and users of animal products. They are also of more fundamental interest: the ability to predict nutritional value from chemical tests is some measure of our understanding of the reasons for the differing nutritional values of individual materials. To throw light on both these aspects a collaborative study was undertaken involving commercial meals with different contents of FDNB-reactive lysine as well as samples prepared in a pilot plant using variables expected to have an influence on the measured lysine values. In order to facilitate the statistical comparisons between the various methods, the various treatments were chosen so as to represent extremes. In addition to the work of the authors of the paper, who are also responsible for the collation of the results, further tests were run in Copenhagen by Dr. B. O. Eggum at the Institute of Animal Science and by Ing. W. Schmidtsdorff at the Ministry of Fisheries Laboratory, and in Bergen by Dr. J. Njaa at the Government Vitamin Laboratory. The test samples were prepared on the pilot plant at SSF and characterized by Mr. N. Urdahl and Mr. E. Nygard. The laboratories are coded V–Z in the tables of results.

MATERIALS AND METHODS

Test Materials. A description of the samples is given in Table I. Fish meals A–F were prepared in a pilot plant,

Department of Applied Biology, University of Cambridge, Cambridge, CB2 3DX, England (K.J.C), and the Norwegian Herring Oil & Meal Industry Research Institute, Bergen, Norway (J.O.).