Protein and Peptide Analysis
Using a Dedicated Amino Acid Analyser

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Compositional analysis

Amino acid analysis has always been a critical tool in protein chemistry as it provides the important initial information for structural analysis. The production of rare proteins and site-specific modifications by recombinant technology has increased the importance of confirming the predicted structure. Hundreds of different amino acids are possible by post-translational modifications and these can often be distinguished from the naturally occurring structures by retention time and 570/440 peak area ratios, for example phosphorylation reduces the retention time and nitration, hydroxylation and iodination reduce the 570/440 ratio.

Complete hydrolysis of the protein to its component amino acids allows automated separation and quantification at the pmole-nanomole level. Although sequence information is lost, compositional data provides a protein specific fingerprint for identification purposes and by summation the amount of protein can be determined.

Information from the genome does not provide information about the functions of many genes and investigations on the process of gene expression do not show the processes of co- and post-translational modification that result in polypeptide modification. If the identity is not known then the compositional figures can be used in searching protein databases.

Information provided by amino acid ion exchange chromatography :

- Accurate quantitation of total protein based on the amino acid composition.
- Fast routine analyses of protein and peptide hydrolysates
- Flexible analysis of specific groups of amino acids such as basic amino acids including tryptophan
- Separation and analysis of a wide range of modified amino acids to follow post-translational changes:
- Analysis of amino sugars in glycosylated peptides
- Ion exchange chromatography enables optional detection systems of ninhydrin and fluorescence.

Ion exchange chromatography

The Biochrom 30 PC controlled liquid ion exchange chromatography system separates amino acids on polymeric cation exchange resin using a sequence of aqueous buffers, which are precisely controlled in composition.

Mechanistically all amino compounds react with ninhydrin via combination of trione groups with amine nitrogen in presence of hydrophilic solvent. After decarboxylation an imine is formed after combination with another molecule of ninhydrin and absorbance is monitored at 570 and 440 nm. A typical output for amino acids and amino sugars is shown below.

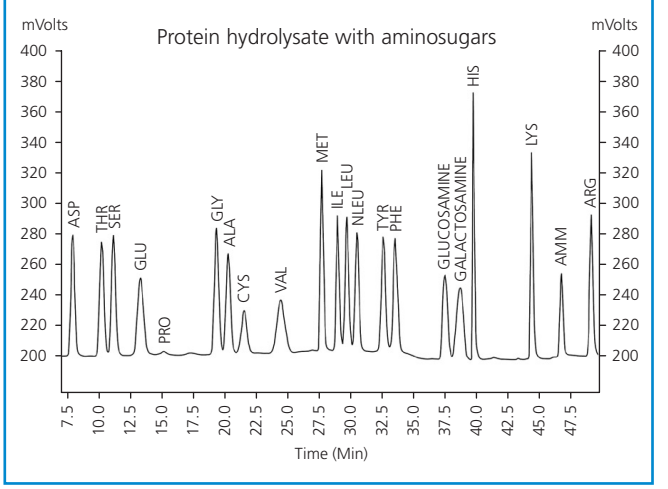
Measurement of absorbance at 2 wavelengths enables characterisation of amino compounds via absorbance ratio as well as retention time.

Amino acid	570/440 ratio		
Phosphoserine	6.00	Cystine	2.14
Taur	6.12	Met	5.21
Phospoeth-anolamine	6.12	Cysteine	1.09
		Leu	5.92
Asp	5.34	Tyr	5.80
Hypro	0.12	Hydroxylysine	2.58
Thr	6.24	Orn	2.92
Asn	4.42	Lys	2.47
Glu	2.99	1-mhis	4.03
Pro	0.18	His	3.90

Peptide bond hydrolysis

Methods are required to maintain progress in identification of large numbers of proteins. Using Biochrom 30 protein hydrolysates can be analysed at the rate of about 45 per day 7 days per week.

Figure 1: Protein identification by database searching.



Sample preparation can take a much longer time than the actual analysis but the correct preparation technique is vital to give an accurate and reliable analysis.

Hydrochloric acid hydrolysis

Undoubtedly the most widely practised method of hydrolysis is in 6M HCl, either by reflux or at 110 °C in a sealed glass tube from which air has been removed by repeated evacuation. The hydrolysis time required for complete liberation of the amino acids depends on the nature of the linkages. Peptide bonds involving the amino acids leucine, isoleucine or valine can require long hydrolysis times to cleave them. For this reason 3 or 4 hydrolyses are often performed with different hydrolysis times to determine the true values for these amino acids.

Rapid Microwave hydrolysis procedure

Acceleration of this step using microwave heating is an attractive possibility but there is little evidence of application to protein mixtures.

A rapid microwave hydrolysis procedure was developed for amino acid determination, employing 6M HCl in sealed vessels system for periods of 5-45 min. This lays the foundations for a systematic study on feed ingredients containing a range of protein contents in the presence of different levels of carbohydrates and lipids.

Protein oxidation prior to hydrolysis

Acid hydrolysis leads to some destruction of the sulphur-containing amino acids. This destruction is particularly important in feedstuff analysis where accurate quantitation of cystine, cysteine and methionine is needed. For this reason, feedstuffs are often oxidised prior to hydrolysis thus allowing accurate quantitation of cysteine and cystine as cysteic acid, and methionine as methionine sulphone.

Sample preparation for free thiol group determinations

These methods do not provide a way to analyse cystine and cysteine separately. This drawback is overcome by reaction of the protein sample with vinylpyridine to convert cysteine residues to pyridylethylcysteine (PEC) before hydrolysis. PEC is stable to the action of 6M HCl during hydrolysis and its analysis is a direct measure of cysteine concentration.

Cysteine residues frequently occur at metal binding sites where the sulphur atoms form covalent bonds with certain metal ions. Cysteine (and serine) frequently has a catalytic role in enzyme active sites

The disulphide group of cystine functions as a means of stabilising structures of many different proteins. This property is also important where cysteine residues occur at other parts of the protein molecule. Bridges formed between spatially adjacent cysteine residues provide extreme cohesive strength.

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The results from analyses on 7 different foods and feedstuffs are shown below. These samples cover a range of protein contents and show the technique to be versatile. In the first column cys was measured in direct hydrolysates. No cysh was detected in these samples, as a result of conversion to cys during acid hydrolysis, in addition to effects caused by degradation.

The remaining columns show results for PEC-derivatised samples. When the reduction step was included total cys and cysh were measured. With no reduction only cysh residues were converted to PEC and cys residues remained underivatised. The final column shows subtraction of cysh from total values and is a measure of cys residues.

Table 1: Percent of cysteine and/or cystine in samples.

Protein	Direct hydrolysis (cys)	PEC & reduction (total)	PEC no reduction (cysh)	total-cysh = cys
Casein	0.56	0.59	0.24	0.35
Fishmeal	0.61	0.87	0.27	0.60
Dried egg	1.23	1.51	0.62	0.89
Soya meal	0.53	0.55	0.15	0.40
Mycoprotein	0.45	0.58	0.20	0.38
Wheatgerm	0.31	0.33	0.18	0.15
Flour	0.12	0.20	0.11	0.09

Methods for the recovery of tryptophan

p-toluenesulphonic acid gives tryptophan values close to the expected integral values, but the procedure cannot tolerate the high concentrations of carbohydrate found in some plant material. Alkaline hydrolysis (4.2 M sodium hydroxide/lithium hydroxide), however, can give complete recoveries of tryptophan but is not suitable for the majority of the other protein amino acids as considerable losses are encountered.

Enzymic digestion of proteins

Several different enzymes are available for specific cleavage of the bonds of a peptide. Proteolytic enzymes such as trypsin, chymotrypsin carboxypeptidase, papain and thermolysin have specific, well-defined activities.

Endopeptidases, for instance pepsin, dipeptidase and aminopeptidase, applied to specific problems in sequencing work. Combinations of these techniques also enable distinction between asp and glu and their amides which are all easily analysed by ion exchange

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