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Review article

Analytical techniques used to study the degradation of proteins and peptides: chemical instability

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Abstract

Instability of peptides and proteins can be divided into two forms: chemical and physical instability. Chemical instability is due to modification/alteration of amino acid residues. There are several types of degradation reactions responsible for this instability. Most frequently described reactions are oxidation, reduction, deamidation, hydrolysis, arginine conversion, β-elimination and racemisation. However, any study of the degradation of a chemical substance lacks reliability when the analytical methodology, that is used is not properly validated. Especially in the investigation, where degradation processes lead to their parent compounds, validation of the analysis is pivotal for the correct interpretation of the results. It is therefore appropriate and useful to assemble an overview of degradation processes in relation to the analytical methods to monitor them. An overview like this can help investigators to make the right

Abbreviations: Ala, alanine; Asn, asparagine; Asp, aspartic acid; Arg, arginine; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; NFK, *N*-formylkynurenine; NBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole; DBD-PyNCS, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole; ACN, acetonitrile; MeOH, methanol; TFA, trifluoracetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IAM, iodoacetamide; IAEDANS, *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl) ethylenediamine; FMOC, 9-fluorenylmethylchloroformate; di-Tyr, di-tyrosine; DABIA, dimethylaminoazobenzene iodoacetamide; *o*-Tyr, *ortho*-tyrosine; iso-Asp, iso-aspartic acid; 2-oxo-His, 2-oxo-histidine; 5-OH-Trp, 5-hydroxy tryptophan; AAA, amino acid analysis; CD, circular dichroism; ECD, electrochemical detection; EI-MS, electron impact-mass spectrometry; Fab-MS/MS, fast atom bombardment-mass spectrometry/ mass spectrometry; FID, flame ionisation detection; GPC, gel permeation chromatography; HPCE, high performance capillary electrophoresis; LC-EC, liquid chromatography-electrochemical detection; LC-MS, liquid chromatography-mass spectrometry; MALDI-MS, matrix assisted laser desorption ionisation-mass spectrometry; NMR, nuclear magnetic resonance; N/PD, nitrogen/phosphor detector; RP-HPLC, reversed phase-high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SIM-GC/MS, selected ion monitoring-gas chromatography-mass spectrometry; UV, ultraviolet.

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choices in their analytical approach of stability problems. The degradation reactions involved in peptide/protein degradation as well as the methods to monitor them are summarized and discussed.

Keywords: Analytical techniques; Degradation; Proteins; Peptides

1. Introduction

Instability of peptides and proteins can be divided into two forms: chemical and physical instability.

Chemical instability is due to modification/alteration of amino acid residues. There are several types of degradation reactions responsible for this instability. Most frequently described reactions are oxidation, reduction, deamidation, hydrolysis, arginine conversion, β-elimination and racemisation. In the literature, the nature and mechanism of these reactions are extensively reviewed. However, elaborate overviews of the analytical methods used to monitor these degradation reactions are hardly available. However, any study of the degradation of a chemical substance lacks reliability when the analytical methodology, that is used is not properly validated. Especially in the investigation, where degradation processes lead to their parent compounds, validation of the analysis is pivotal for the correct interpretation of the results. It is therefore appropriate and useful to assemble an overview of degradation processes in relation to the analytical methods to monitor them. An overview like this can help investigators to make the right choices in their analytical approach of stability problems.

In this paper the degradation reactions involved in peptide/protein degradation as well as the methods to monitor them are summarized and discussed. According to the differences in nature of the reactions they will be considered separately in paragraphs consisting of an introduction about the reaction, overview of the analytical methods used in a tabular, a discussion and conclusion.

The names of the proteins and/or peptides in the tables have been listed in alphabetical order, whereas model peptides without a generic name have been listed in the table under the name 'model peptides'.

2. Oxidation of proteins and peptides

Oxidation is one of the major degradation pathways of protein and peptide pharmaceuticals and takes place under very different conditions. Side chains of the aromatic amino acids His, Tyr, Trp and the sulfur-containing amino acids Met and Cys are sensitive to radiolysis in the presence of oxygen. His and Met are very sensitive for these types of reactions. [1]. The most common amino acids which degrade by metal-catalysed oxidation reactions are His, Met, Cys, Arg, Lys and Pro.

Met can be oxidised to its sulfoxide and, under more drastic conditions, to a sulfone (Fig. 1) [2]. Oxidation of Cys can lead to the formation of inter- or intra-molecular disulfide bridges (Fig. 2) or, under more drastic conditions, to its sulfenic or sulfinic acid and, finally to, sulfonic acid [1–3]. During oxidation of Pro, among other products, 2-pyrrolidone and glutamyl semialdehyde analogues (Fig. 3) are being formed [1,4,5]. Oxidation of aromatic amino acids can lead to the opening of the aromatic side chain: His is, besides some

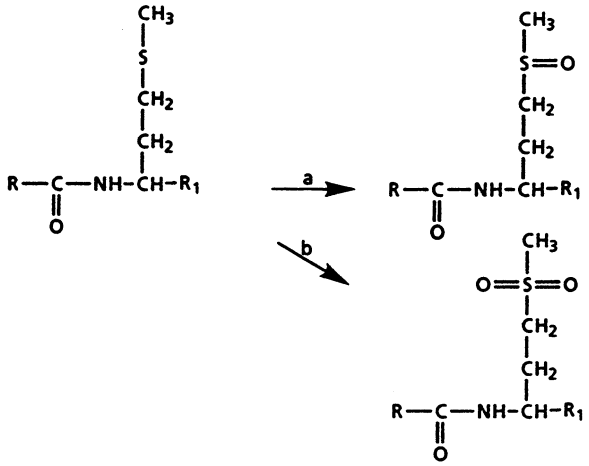


Fig. 1. Oxidation of Met under (a) mild and (b) strong conditions.

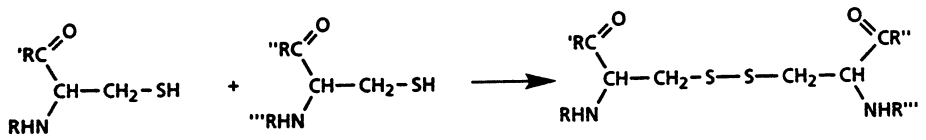


Fig. 2. Oxidation of Cys residues: formation of disulfide bonds.

intermediates, mainly oxidised to 2-oxo-His and Asn [6]. Formation of Asp during His oxidation is probably due to consecutive oxidation of Asn (Fig. 4). As can be seen in Fig. 5, Trp oxidation leads to the formation of several products. The major product formed is *N*-formylkynurenine (NFK) [7]. Besides opening of the aromatic side chain other types of products can also be formed: Tyr can be oxidised to form di-Tyr cross-links at the *o*-position due to the *o*- and *p*-directing hydroxyl group (di-Tyr, Fig. 6). The cross-links can be formed intra- and/or inter-molecularly [2]. Phe oxidation gives rise to the formation of *o*-Tyr (Fig. 7) [8].

The oxidation products show, in comparison with the parent molecules, a change towards more hydrophilicity and polarity: Met to Met sulfoxide or sulfone, Cys to its sulfenic, sulfinic or sulfonic acid, His to its 2-oxo form, Phe to its *o*-Tyr form, Trp to its six oxidation products and Pro to its glutamyl semialdehyde form; on the contrary the formation of intramolecular bonds due to disulfide formation and di-Tyr formation usually causes a decrease in the hydrophilicity and/or polarity of the protein/peptide. Small changes in mass occur in most of the oxidation reactions due to the introduction of oxygen ($\Delta mass = n \times 16$ a.m.u.). Larger changes in mass are seen in the formation of intermolecular disulfide- or di-Tyr bonds. Differences in charge are observed in the oxidation of Cys to its acid forms, His to Asn and Asp, Phe to *o*-Tyr and Trp to kynurenine and dioxindolylalanine due to the generation of carboxylic acid functions. Changes in fluorescent properties can be expected in the oxidation of Trp (decrease in fluorescence [9]) and in the oxidation of Tyr and Phe (increase in fluorescence [8,10]). Alterations in electrochemical properties of amino acid residues can be exploited to monitor the oxidation of His and Phe.

In Table 1, a summary of various peptide oxidation processes and the appropriate analytical techniques used to monitor them are listed.

In studying the oxidation of the various amino acid residues in peptides and proteins chromatographic methods are most widely used as separation techniques. In case of RP-HPLC (gradient and/or isocratic elution for products with oxidation at the Met, His, Tyr, Phe, Trp, Pro and Cys postition) oxidised products elute prior to the corresponding native molecules except for molecules that were oxidised at the His position. This phenomenon is in contradiction with the fact that, in general, oxidation leads to a more hydrophilic product. No explanation is given for this finding. Detection usually takes place with UV, fluorescence, ECD and MS or tandem MS. Measuring the UV absorbance is least specific although it is suitable for the detection of a wide range of products. Techniques like fluorescence, ECD and MS have a more identifying capability. Formation of di-Tyr and *o*-Tyr can specifically be monitored with fluorescence. In case of Trp oxidation conflicting results and conclusions emerge: in various papers [9,13] it is assumed that oxidation products do not exhibit fluorescence. The oxidation product 5-OH-Trp, however, has also been reported to show fluorescence at comparable emission and excitation wavelengths [7] indicating that the parent and the products containing 5-OH-Trp must be separated before analysis. Also the use of ECD is suitable to follow the oxidation of His and Phe into 2-oxo-His and *o*-Tyr, respectively. [12]

MS or tandem MS can be carried out on-line as well as off-line. With these spectroscopic techniques not only the mass difference between the oxidation product and the native molecule can be measured, but also, by interpreting fragmentation patterns, the site of oxidation can be located. In

Table 1
Analytical techniques used to monitor oxidation processes

References	Peptide/protein	Amino acid	Oxidant	Analysis
Biological peptides				
[6]	β-Alanylhistidine	His	Cu^{2+}	AAA after hydrolysis in 6 M HCl/110°C/24 h on a JEOL JLC-300 amino acid analyser Isocratic HPLC on Develosil ODS-5 mobile phase 1% MeOH/0.05 M NH_4Ac for β-alanylhistidine
[11]	Antagonist G	Met	Alkaline media	LC-MS on homepacked Hypersyl ODS, gradient elution, 1.28% ACN min^{-1} with mobile phases 0.1% TFA/10 mM NH_4Ac/ACN/H_2O Fab-MS/MS of selected masses
[6]	*N*-Benzoylhistidine	His	Cu^{2+}	AAA after hydrolysis in 6 M HCl/110°C/24 h on a JEOL JLC-300 amino acid analyser Isocratic HPLC on Develosil ODS-5 mobile phase 3% MeOH/0.05 M NH_4Ac for *N*-benzoylhistidine
[12]	*N*-benzoylhistidine	His	Ascorbate/Cu^{2+}	Isocratic HPLC on TSK-GEL-ODS-80 TM with mobile phase 50 mM NaCl/0.1% heptafluorobutyric acid for *N*-benzoylhistidine and 20% MeOH/50 mM NaCl/0.1% TFA for the acid hydrolysate of oxidised BSA Electrochemical detection on 2-oxo-His at 0.85 V
[12]	Acid hydrolysate of oxidised BSA	His	Ascorbate/Cu^{2+}	Isocratic HPLC on TSK-GEL-ODS-80 TM with mobile phase 50 mM NaCl/0.1%heptafluorobutyric acid for *N*-benzoylhistidine and 20% MeOH/50 mM NaCl/0.1% TFA for the acid hydrolysate of oxidised BSA Electrochemical detection on 2-oxo-His at 0.85V
[5]	BSA	Pro/Lys/Arg/His	Fe^{2+}/O_2, ascorbate/Fe^{2+}/O_2	AAA after acid hydrolysis in 6 M HCl/155°C/45 min on a Dionex Corp. model D-300 AAA Conversion of carbonyl groups studies by treatment with [^{3}H]$NaBH_4$, followed by radioactivity measurements
[13]	Fatty acid free BSA	Tyr/Trp	Peroxynitrite	UV measurements at 330 nm ($\epsilon = 4000$ M^{-1} cm^{-1}) SDS-PAGE, 10% polyacrylamide, silver staining Fluorescence measurements λ_{ex} 325 nm, λ_{em} 410 nm
[9]	Cytochrome *c* peroxidase	Trp	H_2O_2	Fluorescence measurements λ_{ex} 280 nm, λ_{em} 350 nm after denaturation with urea
[14]	EGF	Cys	GSSG/Cys-Cys	HPLC conditions not described MALDI-MS after IAc trapping to characterise which Cys residues are involved in oxidation
[15]	*r*metHuG-CSF	Cys	$CuSO_4$	Gradient HPLC on Vydac C_4 with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O
[16]	Glutathione	Cys	Electrochemical	Isocratic LC-EC on Nucleosil C_{18}, mobile phase not described Electrochemical detection on sulfhydryl groups with CuHCF glassy carbon electrode, potential scanning 0.5–1.0 V
[5]	Glutatione synthetase	Pro/Lys/Arg/His	Fe^{2+}/O_2, ascorbate/Fe^{2+}/O_2	AAA after acid hydrolysis in 6 M HCl/155°C/45 min on a Dionex Corp. model D-300 AAA Conversion of carbonyl groups studies by treatment with [^{3}H]$NaBH_4$, followed by radioactivity measurements

Table 1 (Continued

References	Peptide/protein	Amino acid	Oxidant	Analysis
[10]	Hair protein	Phe	OH radicals	Gradient HPLC on Ultrasphere C_{18} with stepwise gradient, mobile phases 12 mM phosphate (pH 7.2)/ACN/H_2O. Analysis after OPA derivatization Fluorescence detection λ_{ex} 330 nm, λ_{em} 450 nm. Isocratic HPLC on Spherisorb ODS-2 mobile phase 1% ACN.
[6]	Histidyltyrosine	His	Cu^{2+}	AAA after hydrolysis in 6 M HCl/110°C/24 h on a JEOL JLC-300 amino acid analyser Isocratic HPLC on Develosil ODS-5 mobile phase 25% MeOH/0.05M NH_4Ac for histidyltyrosine
[17]	Glycated insulin	His	Cu^{2+}	Amino acid analysis (AAA) after hydrolysis with 6 M HCl/110°C/24 h on a JEOL JLC-300 amino acid analyser Edman degradation Gradient HPLC on Develosil C_{18} with a stepwise gradient combined of the mobile phases 0.5% TFA/MeOH/H_2O and 0.25% TFA/ACN/H_2O UV detection at 215 nm
[18]	α-Lactalbumin	Cys	Dsb^s_s/GSSG	Gradient HPLC on Vydac C_{18}, gradient not described, mobile phases 0.1% TFA/ACN/H_2O UV detection at 220 nm UV detection at 215 nm Site directed mutagenesis to characterise
[19]	Lens protein	Tyr/Phe	Peroxidase/H_2O_2	SIM-GC/MS 30 m DB-5 capillary column, temperature program, 70–290°C, derivatizing agent *N*,*O*-acetyl isopropyl after acid hydrolysis
[8]	Lysozyme	Tyr/Phe	HRP-H_2O_2	Gradient HPLC on Supelcosil LC-318 C_{18} with stepwise gradient, mobile phases 0.1% HFBA/ACN/H_2O. Analysis after acid hydrolysis in 6 M HCl/110°C/24 h Fluorescence detection λ_{ex} 317 nm, λ_{em} 407 nm SIM-GC/MS 30 m DB-5 capillary column, temperature program, 100–290°C, derivatizing agent *N*,*O*-acetyl isopropyl after acid hydrolysis
[5]	Lysozyme	Pro/Lys/Arg/His	Fe^{2+}/O_2, ascorbate/Fe^{2+}/O_2	AAA after acid hydrolysis in 6 M HCl/155°C/45 min on a Dionex Corp. model D-300 AAA Conversion of carbonyl groups studies by treatment with [^{3}H]$NaBH_4$, followed by radioactivity measurements
[18]	Bovine pancreatic trypsin inhibitor	Cys	Dsb^s_s/GSSG	Gradient HPLC on Vydac C_{18}, gradient not described, mobile phases 0.1% TFA/ACN/H_2O UV detection at 220 nm
[20]	pro-αI(III)D1	Cys	Not described	To make distinction between intra- and inter molecular disulfides two dimensional SDS (10% polyacrylamide), first dimension non-reducing, second dimension reducing
[21]	Human relaxin	His/Met	Ascorbic acid/O_2/Cu^{2+} or Fe^{3+}	AAA after acid hydrolysis on a Beckman 6300 amino acid analyser Edman degradation after tryptic digestion (trypsin) Gradient HPLC on RP-C4 Vydac with gradients of 0.81% ACN min^{-1} and 1.62% ACN min^{-1} with mobile phases 0.1% TFA/ACN/H_2O UV detection at 214 nm LC-MS and LC-tandem MS (RP-C_{18}) after tryptic digestion (trypsin) mobile phase 0.1% TFA/ACN/H_2O Fab-MS/MS after tryptic digestion

Table 1 (Continued)

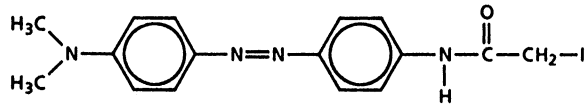
References	Peptide/protein	Amino acid	Oxidant	Analysis
[22]	Human relaxin (Rlx) $AcSWMEE\text{-}NH_2$ (Rlx1) $AcCNH_2\text{-}S\text{-}S\text{-}AcCGM\text{-}STNH_2$ (Rlx2)	Met	H_2O_2	AAA after acid hydrolysis on a Beckman 6300 amino acid analyser Edman degradation after tryptic digestion (trypsin) Gradient HPLC on Nucleosil C_{18} with gradients of 0.68% ACN min^{-1} or 0.9% ACN min^{-1} for Rlx1 and 0.36% ACN min^{-1} or 0.9% ACN min^{-1} for Rlx2 with mobile phases 0.1% TFA/ACN/H_2O UV detection 214 nm LC-MS and LC-tandem MS (RP-C_{18}) after tryptic digestion (trypsin) mobile phase 0.1% TFA/ACN/H_2O Fab-MS/MS after tryptic digestion
[23]	Rhodanese	Cys	O_2	DTNB assay at 412 nm with $\epsilon = 12\,500$ M^{-1} cm^{-1} in SDS media and $\epsilon = 13\,500$ M^{-1} cm^{-1} in non-SDS media
[24]	RNA acetylcholine esterase (32–60)	Cys	H_2O_2	DTNB assay at 412 nm HPCE, electrolyte 20 mM citrate or 50 mM phosphate pH 2.5, 25 kV, 50 μm fused silica UV detection at 200 nm
[8]	RNase	Tyr/Phe	HRP-H_2O_2	Gradient HPLC on Supelcosil LC-318 C_{18} with stepwise gradient, mobile phases 0.1% HFBA/ACN/H_2O. Analysis after acid hydrolysis in 6 M HCl/110°C/24 h Fluorescence detection λ_{ex} 317 nm, λ_{em} 407 nm SIM-GC/MS 30 m DB-5 capillary column, temperature program, 100–290°C, derivatizing agent *N,O*-acetyl isopropyl after acid hydrolysis
[5]	RNaseA RNaseB	Pro/Lys/Arg/His	Fe^{2+}/O_2, ascorbate/ Fe^{2+}/O_2	AAA after acid hydrolysis in 6 M HCl/155°C/45 min on a Dionex Corp. model D-300 AAA conversion of carbonyl groups studies by treatment with [^{3}H]$NaBH_4$, followed by radioactivity measurements
[25]	Phage P22 tailspike	Cys	In vitro/vivo, O_2	Non-reducing SDS-PAGE, 7.5% polyacrylamide
[26]	Trypsinogen	Cys	Not described	Isocratic gel chromatography on Superose 12 with mobile phase 100 mM HAc/100 mM NaCl/2 M urea, flow 0.5 ml min^{-1} UV detection at 280 nm Gradient cation exchange HPLC on ProGel-TSK 5PW with a gradient of 8.75 mM NaCl min^{-1}, mobile phases 25 mM NaAc (pH 4)/2M urea/NaCl/20% ACN HPCE, electrolyte PEG/$NaPO_4$ pH 2.0, 20 kV, 50 μm fused silica. UV detection at 200 nm
[27]	TR-γ	Cys	Cu^{2+}	Gradient HPLC on Vydac C_{18} with gradient 0.5% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O. Analysis after DABIA (Fig. 16) labeling  Fig. 16. Dimethylaminoazobenzene iodoacetamide (DABIA). UV detection at 215 and 540 nm Characterisation after reduction, IAc labeling, tryptic digestion and HPLC with UV detection at 215 and 540 nm

Table 1 (Continued)

References	Peptide/protein	Amino acid	Oxidant	Analysis
Model peptides				
[5]	Poly-L-Arg, Poly-L-Asn, Poly-L-Gly Poly-L-Lys, Poly-L-Pro, Poly-L-Tyr	Pro/Lys/Arg/His	Fe^{2+}/O_2, ascorbate/Fe^{2+}/O_2	AAA after acid hydrolysis in 6 M HCl/155°C/45 min on a Dionex Corp. model D-300 AAA Conversion of carbonyl groups studies by treatment with [^{3}H]$NaBH_4$, followed by radioactivity measurements
[28]	HM, GGGMGGG	Met	AsA/Fe^{3+}, DTT/Fe^{3+}	Isocratic HPLC on Keystone Hypersil ODS with mobile phase 0.025% TFA in 10% ACN, UV detection at 214 nm
[29]	Ac-MDKVLNRY	Met	SOD	LC-MS and LC-tandem MS on Vydac C_{18}, gradient elution with mobile phases 0.1% TFA/ACN/H_2O stepwise gradient (flow 4 μl min^{-1}) Na tryptic digestion (endoprotease Asp-N)
[7]	Single amino acid	Trp	Not described	Gradient HPLC on Nucleosil 123 3-C_{18} with stepwise gradient, mobile phases 0.1% TFA/MeOH/H_2O and 0.1% TFA/ACN/H_2O UV detection at 260 nm Fluorescence detection λ_{ex} 290 nm, λ_{em} 365 nm Foto-diode array detection 200–400 nm
[4]	Z-Pro	Pro	OH radicals	Isocratic HPLC on Develosil ODS-5 with mobile phase 0.1% TFA in 50% MeOH, flow 2.5 ml min^{-1} UV detection at 210 nm EI-MS, Fab-MS and ^{1}H NMR
[16]	DL-Cys, *N*-acetyl-Cys	Cys	Electrochemical	Isocratic LC-EC on Nucleosil C_{18}, mobile phase not described Electrochemical detection on sulfhydryl groups with CuHCF glassy carbon electrode, potential scanning 0.5–1.0 V
[24]	TFQTNPDGTIQFRC	Cys	H_2O_2	DTNB assay at 412 nm HPCE, electrolyte 20 mM citrate or 50 mM phosphate pH 2.5, 25 kV, 50 μm fused silica, UV detection at 200 nm

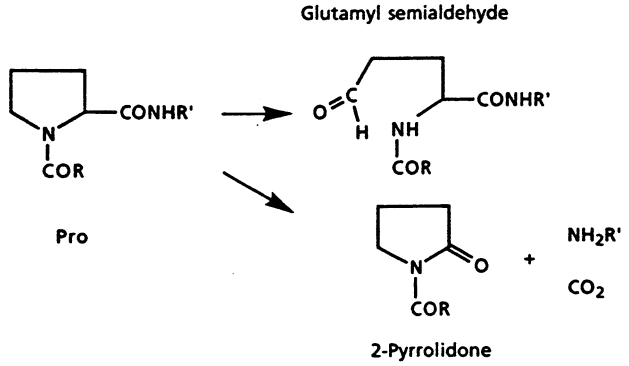


Fig. 3. Oxidation products of Pro.

case of a known amino acid sequence this is a less laborious and more informative method than the Edman degradation followed by amino acid analysis or acid hydrolysis prior to amino acid analysis. Moreover, complications of the Edman procedure are avoided: Trp destruction takes place, Met is formed back from oxidized Met after hydrolysis. Also, in amino acid sequencing using Edman degradation only the first 5–30 amino acids in a protein can be determined. By digesting the protein with restriction enzymes prior to the Edman degradation, the sequence of the whole protein can be determined since fragments containing the oxidation site give information about which amino acid is oxidised.

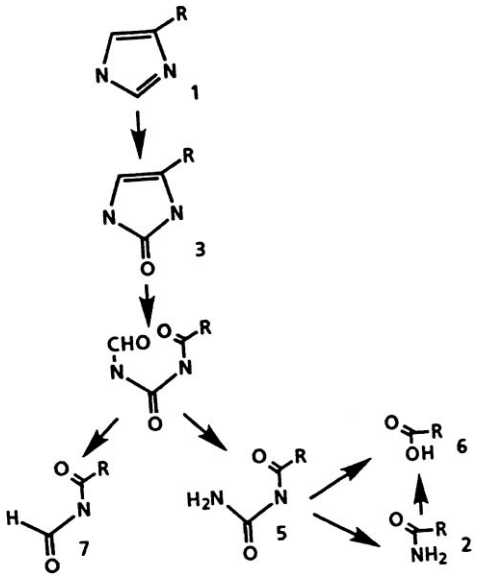


Fig. 4. Ring opening of the imidazole ring of His: (1) His, (2) Asn, (3) 2-oxo-His and (6) Asp, if $R = CH_2–R'$ ($R' =$ amino acid).

SIM-GC/MS is used to monitor the oxidation of Tyr and Phe into di-Tyr and *o*-Tyr, respectively.

Formation of aggregates due to oxidation of Tyr and Cys is chromatographically monitored with SEC, electrophoretically with (one and two dimensional) SDS-PAGE (reducing and non-reducing) and HPCE. With these techniques a distinction between intra- and inter-molecular crosslinks can be made: intra-molecular crosslinks lead to smaller molecules which elute slower in SEC and migrate faster in non-reducing SDS-PAGE as compared to inter-molecular crosslinking.

3. Reduction of proteins and peptides

Reduction of the Cys-Cys moiety in proteins and peptides results in the disruption of intra- or inter-molecular disulfide bonds. The disulfide cross-links play an important role in the stabilisation of the three-dimensional structure of the protein or peptide [30]. Intra-molecular cross-links of cysteines are often required for the folding of the protein or peptide to its biologically active form [31]. For the stabilisation of proteins consisting of monomers (quaternary structure), inter-molecular disulfide bonds are necessary [32].

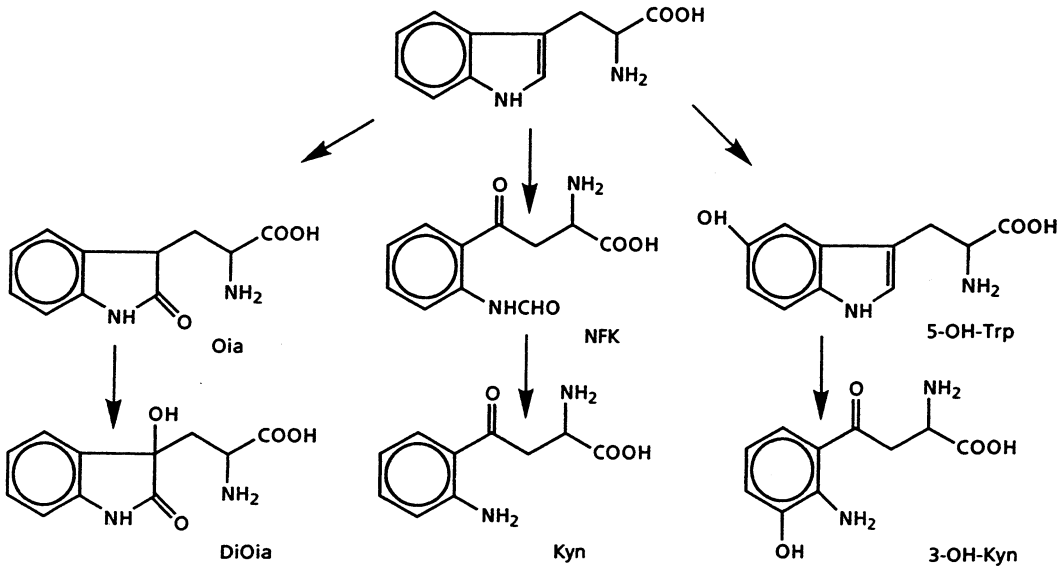


Fig. 5. Oxidation products of Trp. Oia is oxindolylalanine, DiOia is dioxindolylalanine, Kyn is kynurenine and NFK is *N*-formylkynurenine.

Disulfide cross-links can be reduced by various reagents. Widely applied are the thiols β-mercaptoethanol (β-ME) and dithiothreitol (DTT). The reduction with β-ME [33] and DTT [34] depends strongly on the pH. The mercaptide anion rather than the neutral form participates in the displacement reaction with the disulfide bonds. Denaturants like guanidine hydrochloride (GndHCl) and urea can accelerate the reduction. DTT appears to reduce disulfide bonds in proteins quantitatively (Fig. 8) [33]. Reduction of intra- and inter-molecular disulfide bonds can be monitored by following the loss in tertiary structure, change in shape, formation of free sulfhydryl groups, changes in hydrophobicity and charge of the molecule.

A native protein is folded in such a way that the hydrophilic amino acid residues (Asp, Glu, Lys, Arg and His) are largely exposed on the outside of the protein and the hydrophobic amino acids (Phe, Tyr, Trp, Leu, Ile and Val) are confined in the interior of the molecule [35]. The presence of acetonitrile (ACN) and/or methanol (MeOH) in the mobile phase activates the denaturation. Consequently, the protein unfolds, the hydrophobic amino acids are released and become available for interactions with the mobile and stationary HPLC-phases which results in delayed elution compared to the native protein.

Changes in protein shape are caused by the unfolding of the protein/peptide due to disruption

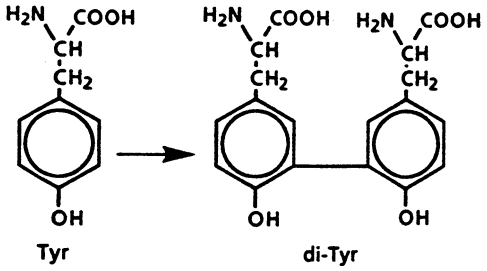


Fig. 6. Oxidation of Tyr to di-Tyr.

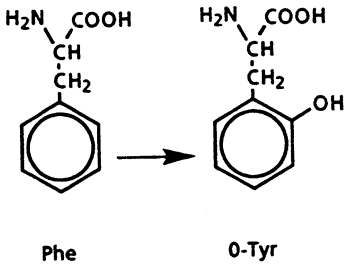


Fig. 7. Oxidation of Phe to *o*-Tyr.

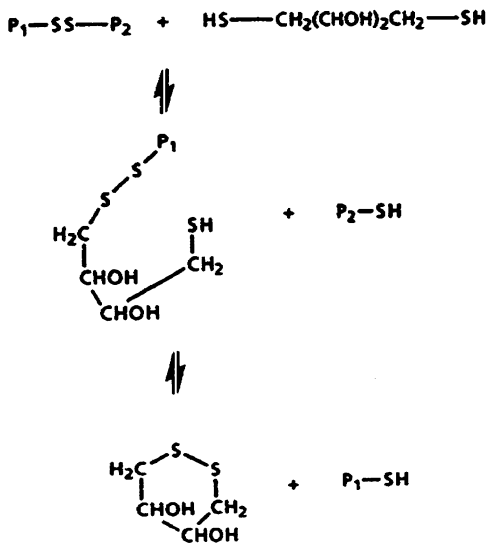


Fig. 8. Reduction of disulfide by DTT.

of intra-molecular disulfides. Disruption of the quaternary structure is due to the cleavage of inter-molecular disulfides and causes, besides changes in shape, also a decrease in molecular weight [32].

Loss of tertiary structure also introduces changes in spectroscopic properties like UV and fluorescence due to exposure of chromophores and fluorophores to a different micro-environment.

In Table 2, a summary of various peptide reduction processes as well as the analytical techniques to monitor these processes is presented.

The two most frequently described techniques in monitoring protein and peptide reduction are RP-HPLC and SDS-PAGE.

In RP-HPLC identification as well as quantitation can be carried out. Due to the presence of physical denaturants like ACN or MeOH in many mobile phases the hydrophobic groups become exposed to the exterior of the molecule. In an RP-HPLC system this results in an increase in capacity factor compared to the native molecule. Quantitation can easily be performed by measuring peak areas obtained by monitoring the UV absorbance or fluorescence signal after chromatographic separation. Other methods which quantify the number of sulfhydryl groups, are the DTNB assay or the radioalkylation assay where [^{14}C]iodoacetamide is used as radioactive label. Both techniques can be used without a separation step preceding analysis. Although the DTNB assay is easier to perform, the radiolabel assay has a lower detection limit. The major drawback of quantitation without the use of a separation technique is that no distinction between intermediates and end products can be made and, therefore is not applicable in stability research.

RP-HPLC determination of the residues after reduction is more laborious. Characterisation of the Cys groups involved in reduction can be carried out with a labeling technique (Fig. 9) using IAEDANS as label (Fig. 10) [39]. This is the only technique known where the site of reduction can be characterised without using synthetic analogs.

In studying reduction processes of peptides and proteins, SDS-PAGE is also very widely used. This electrophoretic technique, however, can not be used for quantitation. On the other hand it is very useful in the characterisation of the nature of the reduction. Distinction can be made between the reduction of an intra-molecular and an inter-molecular disulfide bond. Also the number of sulfhydryl groups involved in reduction can be measured using iodoacetamide as label to introduce an extra charge in the molecule at sites where new sulfhydryl groups are formed.

The use of fluorescence and CD in these studies is less useful. Fluorescence only provides information about the (un)folding of the peptide/protein. With CD only changes in secondary or tertiary structure can be seen. However, during reduction the secondary structure will not change significantly. Changes in tertiary structure are due to physical instability of the molecule and are not necessarily related to the reduction of disulfides.

4. Conversion of arginine into ornithine and/or citrulline

Exposure of proteins and peptides to alkali induces various degradation pathways among which the conversion of Arg into ornithine and, possibly, citrulline. The conversion of the guanidino side chain of Arg to form ornithine and citrulline is depicted in Fig. 11 [35].

Table 2
Analytical techniques used to monitor reduction processes

Reference	Peptide/protein	Reductans	Analysis
[36]	C-9	DTT	Radioalkylation assay, [^{14}C]iodoacetamide (6.3×10^6 cpm mmol^{-1})
[31]	DsbC	GSH	Gradient HPLC on Vydac C_{18} with gradient 0.46% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O UV detection at 220 nm SDS-PAGE, high pH denaturating gel, 10% poyacrylamide 8 M urea, Coomassie Blue staining SDS-PAGE carried out to determine the charge after iodoacetic modification
[34]	*r*metHuG-CSF	DTT	Gradient HPLC on Phenomenex W-Porex 5 C_4 with gradient 0.7% ACN min^{-1}, mobile phases 10 mM $HClO_4$/100 mM $NaClO_4$/ACN/H_2O UV detection at 205 nm
[37]	HGPI	DTT	CD, far-UV 200–260 nm with 1 mm UV cell, near-UV 230–350 nm with 10 mm UV cell Fluorescence measurements on Tyr, λ_{ex} 280 nm, λ_{em} 335 nm
[38]	Hen egg-white lysozyme	2-Aminophenol, β-ME	Gradient HPLC on Wakopak 5 C_{18}, gradient not described, mobile phases 0.1% HCl/ACN/H_2O. Analysis after reduction, alkylation with maleimide, tryptic digestion UV detection at 210 nm Characterisation after fraction collection from HPLC, AAA after acid hydrolysis in 6 M HCl/0.1% phenol/150°C/1 h on a Hitachi L-8500 amino acid analyser
[39]	Ovalbumin	DTT	Gradient HPLC on Cosmosil 5 C_4-RA300 with gradient 1% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O Fluorescence detection λ_{ex} 340 nm, λ_{em} 520 nm Characterisation: procedure order, labelling with iodoacetamide, reduction, labelling with IAEDANS, tryptic digestion, gradient HPLC with fluorescence detection SDS-PAGE, high pH denaturating gel, 10% poyacrylamide 8 M urea, Coomassie Blue staining SDS-PAGE carried out to determine the charge after iodoacetic modification
[38]	Ribonuclease A	2-Aminophenol, β-ME	Gradient HPLC on Wakopak 5 C_{18}, gradient not described, mobile phases 0.1% HCl/ACN/H_2O. Analysis after reduction, alkylation with maleimide, tryptic digestion UV detection at 210 nm Characterisation after fraction collection from HPLC, AAA after acid hydrolysis in 6 M HCl/0.1% phenol/150°C/1 h on a Hitachi L-8500 amino acid analyser
[38]	Soybean trypsin inhibitor	2-Aminophenol, β-ME	Gradient HPLC on Wakopak 5 C_{18}, gradient not described, mobile phases 0.1% HCl/ACN/H_2O. Analysis after reduction, alkylation with maleimide, tryptic digestion UV detection at 210 nm Characterisation after fraction collection from HPLC, AAA after acid hydrolysis in 6 M HCl/0.1% phenol/150°C/1 h on a Hitachi L-8500 amino acid analyser
[30]	SR-Ca^{2+}-AT-Pase	DTT	DTNB-assay at 421 nm with $\epsilon = 13\,600$ M^{-1} cm^{-1}
[32]	*T*.AChE	DTT	Gelfiltration on TSK SW3000 with mobile phase 5 M GndHCl UV detection at 280 nm SDS-PAGE, 7.5% polyacrylamide, reducing as well as non-reducing electrophoresis, staining method not described

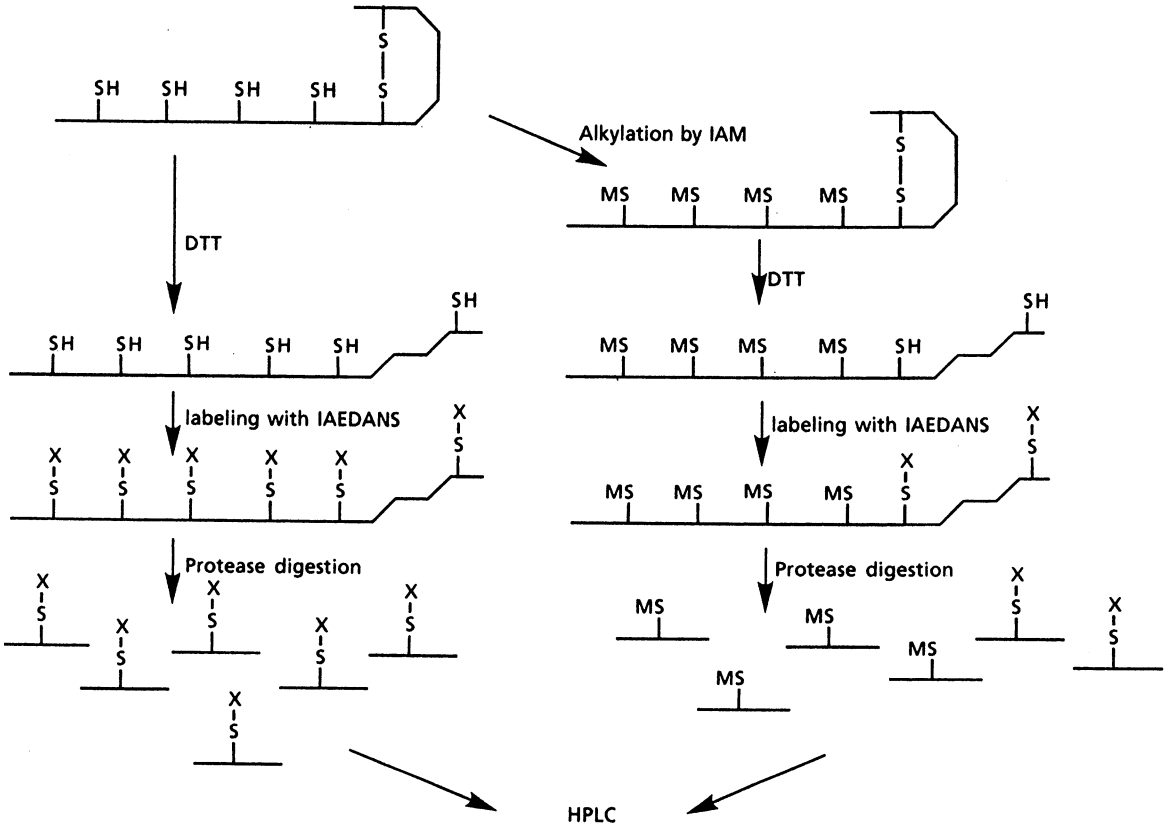


Fig. 9. Characterisation of the sulfhydryls involved in disulfide bonds: labelling with IAEDANS.

The reaction can be studied by observing the mass decrease for ornithine formation ($\Delta m = -42$ a.m.u.), and a mass increase for citrullineformation ($\Delta m = +1$ a.m.u.), a decrease in pI (the guanidino group, pK_a 12.5, is replaced by an aliphatic amine in the formation of ornithine and replaced by an amide in the formation of citrulline) and a change in hydrophobicity.

In Table 3, several analytical techniques used to monitor the conversion of Arg into ornithine and/or citrulline are summarized.

As can be derived from Table 3, the conversion of Arg into ornithine and/or citrulline is poorly described. Both RP-HPLC for studying the conversion of Arg in peptides and GC for conversion of Arg as a single amino acid followed by MS

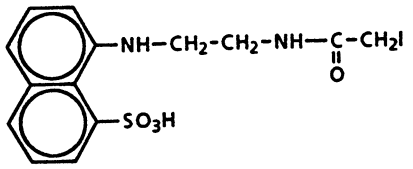


Fig. 10. *N*-iodoacetyl-*N'*-(5-sulfo-1-naphtyl) ethylenediamine (IAEDANS).

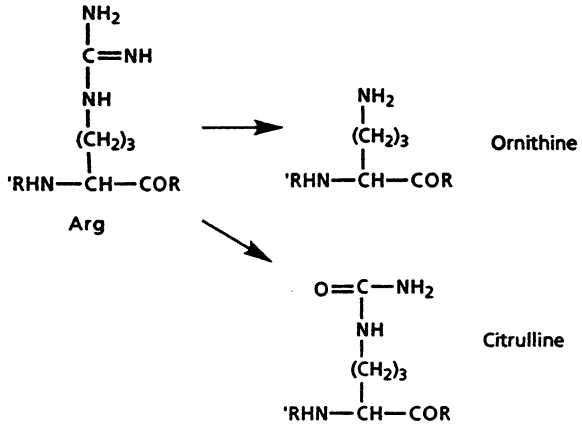


Fig. 11. Conversion of Arg into citrulline and ornithine.

Table 3
Analytical techniques used to monitor conversion processes

Reference	Peptide/protein	Analysis
[11]	Antagonist G	Gradient HPLC on homepacked Hypersil ODS with gradient 1.35% ACN min^{-1}, mobile phases 0.1% TFA/10 mM ammonium acetate/ACN UV detection at 214 nm MS detection Fab-MS/MS for fragmentation patterns
[40]	Arginine	Gradient HPLC on Varian C_{18} Aminotag column (308 K) with stepwise gradient, mobile phases 25% ACN/75% 10 mM tetramethylammonium chloride, 20 mM sodium citrate and 72% ACN/28% 10 mM tetramethylammonium chloride, 20 mM sodium citrate, 10% methanol UV detection at 264 nm Fluorescence detection $\lambda_{ex} = 264$ nm, $\lambda_{em} = 313$ nm
[41]	Arginine	Gradient HPLC on Waters μ Bondapak C_{18} column with mobile phases containing 1-propanol/17 mM sodium citrate, 0.5% SDS. Gradient not described Cation exchange chromatography on Zorbax 300-SCX column TLC on cellulose (Eastman Kodak) with mobile phase *n*-butanol/acetone/diethylamine/water (70:70:14:35) or TLC on Whatmann silica gel plates with mobile phase acetonitrile/acetic acid/water (4:1:1) Detection radioactivity measurement
[42]	Arginine	Cation exchange chromatography on Zorbax 300-SCX column Detection radioactivity measurement
[43]	Arginine	GC/MS on Dowex 1-X8, derivatisation with methyl-8/ACN/methanol Temperature gradient from 140 to 252°C

detection seem to be accurate enough to monitor this reaction.

The cation exchange chromatography with radioactively labelled free L-Arg can be carried out if the disappearance of Arg instead of the formation of the degradation products is the focus of interest. For the characterisation of the site and nature of conversion Fab-MS/MS of the whole peptide fragment or GC/MS after acid hydrolysis yields unequivocal results.

5. Deamidation of asparagine, glutamine and the C-terminal amides

Deamidation at the amide side chain of Asn or Gln residues to form a free carboxylic acid may occur via either direct hydrolysis or via cyclic imide formation. Deamidation of the N-terminal end of a peptide or protein also occurs [2,44,45]. Asn is deamidated more easily than Gln. Deamidation is thought to proceed via the intramolecular nucleophilic attack of the neighbouring nitrogen at the C-terminal side of the Asn residue to form a five-membered cyclic imide intermediate, that spontaneously hydrolyses to give a mixture of Asp and iso-Asp. Gln undergoes a similar deamidation reaction via a six-membered cyclic imide ring [2]. D-Amino acid analogs may originate from the racemization through cyclic imide formation [45]. The reaction scheme is depicted in Fig. 12.

Changes in hydrophobicity and polarity (an amide is replaced by a carboxylic acid), mass ($\Delta m = +1$ a.m.u.), charge (introduction of an acidic side chain instead of a neutral side chain) and formation of a carboxylic acid instead of an amide as well as the formation of ammonia can be used to monitor the deamidation reaction.

In Table 4, a summary of a number of deamidation processes and the techniques used to monitor them is given.

Changes in hydrophobicity and polarity can be exploited relatively easily with RP-HPLC. Small changes caused by deamidation can be monitored with isocratic RP-HPLC whereas larger changes are preferably monitored by using gradient elution. With HPLC quantitation is carried out using UV detection.

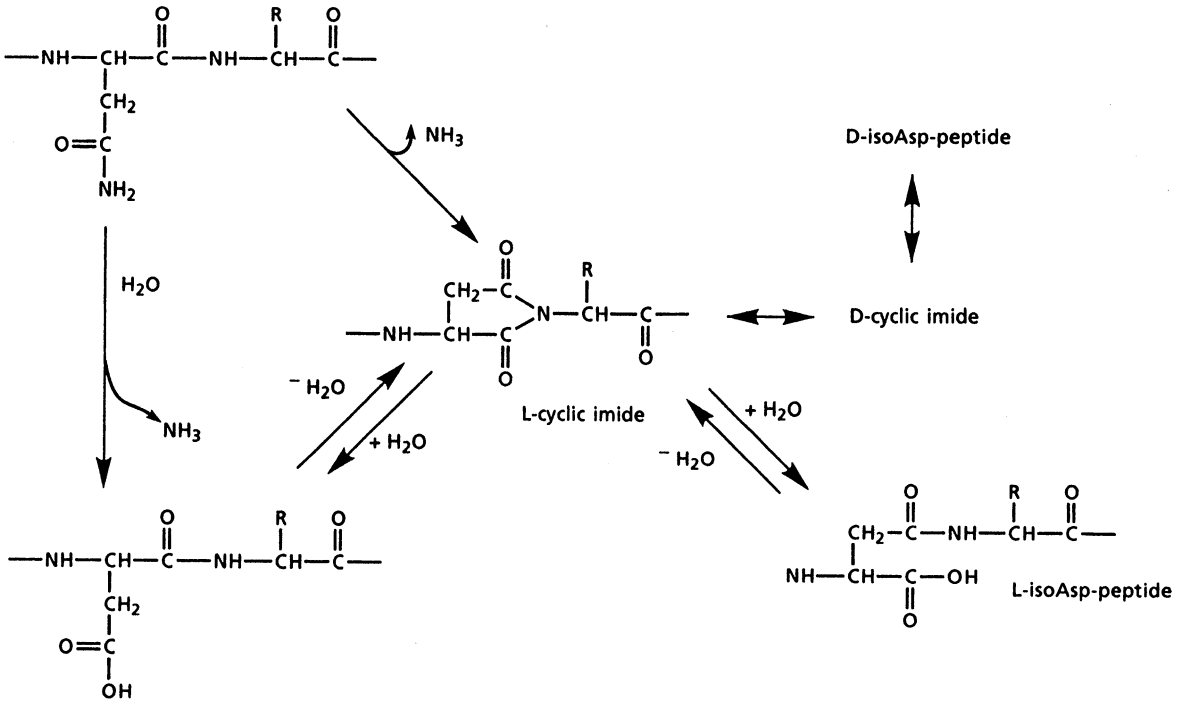


Fig. 12. Deamidation mechanism.

Another direct way of quantifying the deamidation is by measurement of the formation of ammonia. The two described methods, enzymatic ammonia assay and ammonia selective electrode measurement are indirect: sample preparation has to be carried out. In both cases these preparations are very destructive. Moreover, the ammonia assay is a very labour-intensive method.

Differences in mass to charge (m/z) are used in MS detection. An increase of 1 a.m.u. per deamidation site can be measured on-line after HPLC, or off-line. With electrospray as ionisation technique, usually only the mass difference is detected (no fragmentation of the peptide occurs). Fragmentation patterns, obtained with Fab-MS/MS or collision-induced Fab-MS, of the native peptide compared to that of the deamidated peptide give information about the position of the amino acid where the deamidation took place if the amino acid sequence is already known.

Charge-to-mass ratio changes are detected by electrophoresis. In the HPCE-methods UV detection is used. With HPCE in uncoated capillaries, which is a fast and simple method, the introduction of an extra negative charge into the molecule can be derived from an increased retention time of the product. In case of a reversely charge capillary the retention time will be decreased. In isoelectric focussing differences in charge are the only determinants of separation. With this technique the pI of the native and deamidated products can exactly be determined. Isoelectric focussing can be carried out in the conventional way on gels or in capillaries using HPCE technology. The information obtained via isoelectric focussing is a qualitative one.

Electrophoresis with SDS-PAGE gives almost no information about deamidation. Although the negative charge increases, the excess of SDS will cause such an excess of negative charge on the analyte that the increase of only a few negative charges will not be detected. The only valuable information obtained by SDS-PAGE is whether the deamidation also effects the tertiary structure of the peptide/protein dramatically.

Table 4
Analytical techniques used to monitor deamidation processes

Reference	Peptide/protein	Analysis
Biological peptides		
[46,47]	ACTH	Ammonia assay. After sample preparation, NH_3 is used in GLDL-assay to produce NAD and glutamate UV measurement at 340 nm IEF pH range 3–10 on 29.1% acrylamide/0.9% *N*,*N*-methylenebisacrylamide. Coomassie briliant blue G staining
[11]	Antagonist G	Gradient HPLC on homepacked Hypersil ODS with gradient 1.35% ACN min^{-1}, mobile phases 0.1%TFA/10 mM ammonium acetate/ACN UV detection at 214 nm MS detection (electrospray) Fab-MS/MS for fragmentation patterns HPCE, electrolytes varying in pH 6.5, 9.0 and 12.7, varying voltage, 50 and 75 μm fused silica UV detection at 214 nm
[48,49]	Milk casein	Ammonia selective electrode. Deamidated sample dissolved in TCA, ammonia stays in solution, proteins precipitate
[48,49]	Wheat gliadin	Ammonia selective electrode. Deamidated sample dissolved in TCA, ammonia stays in solution, proteins precipitate
[50]	hGRF	Gradient HPLC on VYDAC C18 with gradient 0.5% ACN min^{-1}, mobile phases 0.05% TFA/ACN/H_2O UV detection at 215 nm MS detection (electrospray) HPCE, electrolyte 50 mM citric acid pH 3.0, 15 kV, 50 μm fused silica, UV detection at 215 nm
[51]	*r*-Hirudin	HPCE, electrolyte 60 mM acetate/0.3% PEG 20000/0.1 mM $ZnCl_2$ pH 4.4, 25 kV, 50 μm CElect H_2-coated capillary or 50 μm fused silica capillary UV detection at 214 nm
[52]	HPr	Gradient HPLC on Serva RP8 with a gradient 0.4% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O UV detection at 230 nm IEF, pH range 3–10 (conditions not described) Two dimensional NMR: TOCSY, DQF-COSY and NOESY
[53]	HPr	IEF denaturating tube gels, pH range 6–12 on urea-Nonidel IEF gels. Coomassie brilliant blue R-250 staining IEF (flat polyacrylamide, home made), pH range 4–6. Coomassie brilliant blue R-250 staining
[54]	IL-1β	Gradient HPLC on Vydac 214TP54 silica C_4 with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O IEF, pH range 3.5–9.5 on Ampholine PAG plates (horizontal), densitometric detection Non-reducing SDS-PAGE 8–25% gradient Phastgels, densitometric detection
[55]	*r*IL-2	Gradient HPLC on Nucleosil 5 C_{18} with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O. With or without tryptic digest (Achromobacter protease I) UV detection at 210 and 215 nm IEF, pH range 3.5–9.5 on Ampholine PAG plates. Coomassie brilliant blue R-250 staining Reducing SDS-PAGE on 13.5% polyacrylamide. Coomassie brilliant blue R-250 staining
[48,49]	Egg lysozyme	Ammonia selective electrode. Deamidated sample dissolved in TCA, ammonia stays in solution, proteins precipitate

Table 4
Analytical techniques used to monitor deamidation processes

Reference	Peptide/protein	Analysis
[56]	Cytosolic serine hydroxymethyltransferase	Reducing SDS-PAGE on 12% polyacrylamide
		Denaturation and non-denaturating IEF
		Western blotting
		Densitometric detection at 600 nm in all cases
[48,49]	Soy protein	Ammonia selective electrode. Deamidated sample dissolved in TCA, ammonia stays in solution, proteins precipitate
[57]	Various recombinant DNA derived proteins	HPCE, pH varying between 2.5 and 6.5 (electrolyte composition not described), 8 or 12 kV, 25 μm covalently bonded linear polymer capillary
		UV detection at 200 nm
Model peptides		
[58]	Various peptides derived from substance P	Isocratic HPLC on Nucleosil 120-5 μm, C_{18} mobile phase 0.01 M NaH_2PO_4/0.15 M $NaClO_4$ and different ACN concentrations for the various peptides
		UV detection at 220 nm
[59]	TNSY	Isocratic HPLC on Econosphere C-18, mobile phase 0.1%TFA in 7% ACN
		UV detection wavelength not described
		Fab CID-MS, collision gas xenon
[60]	NGG	Gradient HPLC on RP-C_{18} (type not described), gradient 1.3% ACN min^{-1}, mobile phases 0.05%TFA/ACN/H_2O
		UV detection at 214 nm
[61]	VYPNGA, VYPNCA	Gradient HPLC on Econosphere C_{18} with gradient of 1.3%ACN min^{-1}, mobile phases 0.1%TFA/ACN/H_2O
	VYHNCA, VYHNPA	UV detection wavelength not described
	VYHNGA, VYPNPA	Fab-MS
[62]	VYPNGA	Isocratic HPLC on Econoshere C_{18}, mobile phase 0.1% TFA/7% ACN/H_2O
		UV detection at 214 nm
[63]	VYXNYA	Isocratic HPLC on Econosphere C_{18}, mobile phases of 0.1% TFA and varying concentrations ACN/H_2O depending on the nature of amino acid X
		UV detection at 214 nm
[64]	Ac-GNGG-NHMe	Isocratic HPLC on C_{18} (specifications not described), mobile phase 0.01 M H_2SO_4/KOH pH 3.6
		UV detection at 220 nm

The difference in chemical shift between a nitrogen-bound and an oxygen-bound proton can be used in NMR to monitor deamidation. The use of two-dimensional NMR in this case can also be very useful if the amino acid sequence is not known, which contrasts collision-induced MS or tandem MS.

Quantitative data are preferably obtained via HPLC or HPCE with short analysis time and few sample preparation steps. To determine the qualitative effects of deamidation the discussed techniques all may contribute.

6. Hydrolysis of proteins and peptides

Hydrolysis occurs at the amide bond of the peptide backbone. If the peptide contains an Asp residue the molecule is more sensitive to acid-catalysed hydrolysis than in molecules without Asp residues [2,45]. The hydrolysis can take place at either the N- or C-terminal bond adjacent to the Asp residue. Due to the higher basicity of the Pro nitrogen the peptide bond of an Asp-Pro residue is less stable than bonds between Asp and other amino acids since proto-

nated Pro is a good leaving group. The cleavage of the peptide bond at the C-terminal end of Asp also involves reversible isomerisation between the Asp and iso-Asp forms via the cyclic imide intermediate. This reaction proceeds particularly fast when the peptide possesses an Asp-Gly bond. Peptides containing N-terminal residues neighbouring to Ser and Thr are also sensitive to hydrolysis. In Fig. 13 the reaction is shown.

Changes in mass and size (hydrolysis yields shorter peptide chains), charge (introduction of new carboxylic and amino groups), hydrophobicity, polarity, UV absorption and fluorescence and tertiary structure are used to monitor the hydrolysis.

In Table 5, a summary of the various hydrolytical processes and the analytical techniques used to monitor them, is given.

Isocratic and gradient HPLC are very useful to monitor the hydrolysis providing quantitative data. Detection is carried out UV spectrophotometrically (single wavelength and photodiode array) or electrochemically. In HPCE changes in charge-to-mass ratio due to hydrolysis are detectable. Both HPLC and HPCE are fast methods to quantify the reaction. To get more qualitative information GPC and SDS-PAGE can be used. With both techniques changes in size can be determined. A requisite, however, is that the change in size must be large enough (depending on the conditions and the technique used). In GPC the hydrolysis product will have a longer retention time while in SDS-PAGE the product will have a higher migration velocity. Although both methods give information about the mass of the formed products, the most reliable and precise mass values are obtained with MS. SDS-PAGE and GPC are useful to monitor the hydrolysis reaction, while MS can provide the site of hydrolysis (in case the amino acid sequence of the native molecule is known).

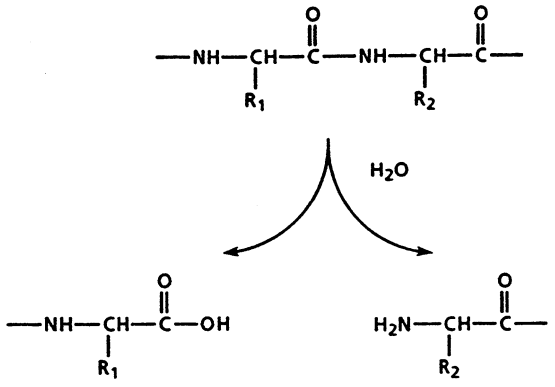


Fig. 13. Hydrolysis of the peptide backbone.

7. Racemisation

The racemisation mechanism of amino acids is thought to proceed via the removal of the α-methine hydrogen by the hydroxide ion. The carbanion intermediate is stabilised by electron-withdrawing side groups such as the side chains of Tyr, Ser and Phe. Asp and Glu racemise very rapidly. Racemisation of both amino acids occurs via a cyclic intermediate: a five-membered cyclic imide for Asp and a six-membered cyclic imide for Glu. The racemisation rate is affected by inductive effects, intramolecular solvation, intramolecular base action and steric hindrance to solvation [2,45]. The racemisation mechanism is given in Fig. 14.

Racemisation in peptides and proteins causes formation of diastereomers which can be distinguished from one another based on differences in physico-chemical properties like hydrophobicity and polarity. If the racemisation is carried out in aqueous solutions no changes in charge and mass do occur. Racemisation in deuterated media gives rise to a mass increase of $+1$ a.m.u. for each amino acid racemised. The introduction of deuterium changes the nuclear magnetic resonance properties. For enantiomers as well as diastereomers a change in optical rotation is also obvious.

In Table 6, a summary of analytical techniques used to monitor racemisation processes in peptides is given.

All analytical techniques used in studying the racemisation are dependent on the use of chiral

Table 5
Analytical techniques used to monitor hydrolytic processes

Reference	Peptide/protein	Analysis
Biological peptides		
[65]	Antagonist G	Isocratic HPLC on homepacked Hypersil ODS with mobile phase 10 mM perchloric acid/100 mM sodium perchlorate/45% ACN/H_2O UV detection at 214 nm HPCE, electrolyte 75 mM borate pH 9.0, 25 or 30 kV, 50 μm fused silica HPCE, electrolyte 10 mM phosphate/0.14 mM Fluorad pH 6.5, −12.5 kV, 75 μm fused silica UV detection at 214 nm
[66]	Bradykinin	High resolution time-of-flight plasma desorption MS
[67]	Goat β-casein	SDS-PAGE 15% polyacrylamide. Coomassie Brilliant Blue R-250 staining Densitometric detection at 633 nm
[66]	Desmopressin	High resolution time-of-flight plasma desorption MS
[68]	CTL-epitopes	Gradient HPLC on DeltaPak C_{18}-300 Å with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O Detection not described
[69]	*r*hIL-11	Gradient HPLC on Vydac C_4 with gradient 3.6% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O UV detection at 280 nm Laser desorption time-of-flight MS. Analysis after fractionation of degradation products with HPLC
[70]	Max-protein	Matrix assisted laser desorption/ionization MS after trytic digestion (V8)
[71]	Substance P analogues	Gradient HPLC on μ-Bondapak C_{18} with gradient 2% ACN min^{-1}, mobile phases *o*-phosphoric acid/TEA (pH 2.5)/ACN/H_2O Isocratic HPLC on μ-Bondapak C_{18} with mobile phase 0.15% TFA/10 mM ammonium acetate/46% ACN/H_2O Electrochemical detection
[72]	Soy bean protein	Gradient HPLC on Spherisorb ODS-2 with stepwise gradient, mobile phases TFA/ACN/H_2O UV detection at 220 nm Isocratic gel permiation HPLC on ZorbaxBio GF-250, separation range 4000–400 000, mobile phase 0.1 M phosphate, 0.1% SDS (pH 8.0) UV detection at 214 nm
[73]	Various peptides	^{1}H NMR Two dimensional TLC
[74]	Various peptides	Gradient HPLC on Hypersil ODS bonded phase with gradient 2.1% ACN min^{-1} and 2% MeOH min^{-1}, mobile phases 0.05 M sodium acetate/ACN/MeOH Photodiodearray detection
Model peptides		
[75]	Gly-Gly	Gradient HPLC on Spherisorb ODS-2 with gradient 0.33% ACN min^{-1}, mobile phases 4% DMF/25 mM sodium acetate/ACN/H_2O UV detection at 275 nm SDS-PAGE on 10% polyacrylamide. Coomassie Brilliant Blue staining
[76]	LMW-peptides	Capillary ITP, leading electrolyte 0.01 M HCl/ethanolamine pH 9.5, terminating electrolyte 0.01 M Ala/BaOH pH 10, 100 μA, 0.7 × 80 mm capillary electrophoresis tube UV detection at 200 nm Potential gradient detection
[77]	LWMRFA, RVYIHPFHL, EADKADVNVLTKAKSE, GIGKFLHSAGKFGKAVGEIMKS	Fab-MS and ESI-MS

Table 6
Analytical techniques used to monitor racemisation processes

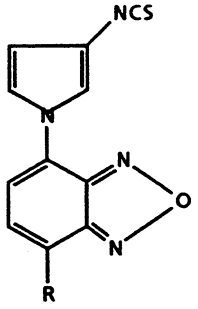
Reference	Peptide/protein	Amino acid	Analysis
Biological peptides			
[11]	Antagonist G	Met	Gradient HPLC on homepacked Hypersil ODS with gradient 1.35% ACN min^{-1}, mobile phases 0.1% TFA/10 mM ammonium acetate/ACN UV detection at 214nm MS detection (electrospray) GC on Chirasil-L-Val, N/PD detection, temperature gradient 75–200°C, derivatization with 2-propanol and TFA/ethyl acetate, H_2 carrier gas Analysis after acid hydrolysis (6 M HCl/110°C/24 h) Optical rotation on micropolarimeter, wavelength λ 589 nm
[79]	Human articular cartilage	Asp	GC on L-Valine-*S*-phenyl-α-ethylamide linked to hydrolyzed XE-60, FID detection. Derivatization with trifluoroacetyl. Temperature gradient 40–220°C Analysis after acid hydrolysis (6 M HCl/100°C/6 h)
[80]	αB-crystallin	Asp	Gradient HPLC on NovaPak ODS with gradient 0.33% ACN min^{-1}, mobile phases 0.1 M acetate pH 6.0/3% THF/ACN/H_2O Fluorescence detection λ_{ex} 344 nm λ_{em} 433 nm Analysis after acid hydrolysis (6 M HCl/108°C/7 h) and derivatization with OPA and Boc-L-Cys
[81,82]	Dentin proteins	Asp	GC on Chirasil-L-Val, FID detection H_2 carrier gas. Derivatization with isopropanol and TFA Analysis after acid hydrolysis (6 M HCl/100°C/6 h)
[83]	[D-Ala^2,D-Leu^5]-enkephaline	Pro/Phe/Leu/Val	Gradient HPLC on Intersil ODS-80A with stepwise gradient, mobile phases 0.05% TFA/ACN/H_2O Fluorescence detection λ_{ex} 490 nm λ_{em} 530 nm for NBD-PyNCS (Fig. 17) and λ_{ex} 450 nm λ_{em} 560 nm for DBD-PyNCS  Fig. 17. NBD-PyNCS. Analysis after derivatization with NBD-PyNCS and DBD-PyNCS
[84]	Human fossil bone	Asp	Gradient HPLC on Synchropak RP-P C_4 with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O UV detection at 215 nm Analysis after acid hydrolysis (6 M HCl/150°C/20 min) and OPA derivatization GC on Chirasil-L-Val, experimental conditions not described
[10]	Hair protein	Hydroxy-Pro	Gradient HPLC on XLODS reversed phase with stepwise gradient, mobile phases 12.5 mM sodium phosphate/1% THF/ACN/H_2O Fluorescence detection λ_{ex} 330 nm λ_{em} 450 nm Analysis after derivatization with NBD-Cl
[85]	Human lens protein	Ser/Asp/Lys/His/Ala/Glu/Ile/Leu	Isocratic HPLC on Waters C_{18} with mobile phase 5% MeOH UV detection at 400 nm Optical dispersion, UV region 260–400nm Analysis after hydrolysis NMR. One dimensional NMR Analysis after HPLC fractionation

Table 6 (Continued)

Reference	Peptide/protein	Amino acid	Analysis
[83]	Morpholine tolerance peptide	Pro/Phe/Leu/Val	Gradient HPLC on Intersil ODS-80A with stepwise gradient, mobile phases 0.05% TFA/ACN/H_2O Fluorescence detection λ_{ex} 490 nm λ_{em} 530 nm for NBD-PyNCS (Fig. 17) and λ_{ex} 450 nm λ_{em} 560 nm for DBD-PyNCS Analysis after derivatization with NBD-PyNCS and DBD-PyNCS
[83]	Neurotensin	Pro/Phe/Leu/Val	Gradient HPLC on Intersil ODS-80A with stepwise gradient, mobile phases 0.05% TFA/ACN/H_2O Fluorescence detection λ_{ex} 490 nm λ_{em} 530 nm for NBD-PyNCS and λ_{ex} 450 nm λ_{em} 560 nm for DBD-PyNCS Analysis after derivatization with NBD-PyNCS and DBD-PyNCS
[86]	Resin	All amino acids	Gradient HPLC on μBondaPak C_{18} with stepwise gradient, mobile phases 0.1% TFA/ACN/H_2O Isocratic HPLC on μBondaPak C_{18}, mobile phase 20 mM sodium acetate pH 4.0/8% ACN UV detection at 340 nm Analysis after DNPA derivatization
[87]	Various protected peptides	Glu/Thr/Ile	Gradient HPLC on LiChrosorb RP18 with gradient 1.6% ACN min^{-1}, mobile phases 0.1% TFA/ACN/H_2O UV detection at 254 nm Analysis after derivatization with TMD
[88]	Various peptides	All amino acids	Gradient HPLC on PhaseSep ODS, gradient not described, mobile phases ACN/2-propanol/40 mM triethylaminephosphate/40 mM Titriplex pH 2.3 UV detection at 338 nm Analysis after acid hydrolysis (6 M HCl/110°C/24 h) and derivatization with Marfey's reagent (Fig. 18)
[78]	Various peptides	Asp/Glu/Asn/Gln	Gradient HPLC on RP-C_{18} (column specifications not described) with gradient 2.3% MeOH min^{-1}, mobile phases sodium acetate/MeOH/H_2O Fluorescence detection, wavelenghts not described Analysis after enzymatic hydrolysis, derivatization with OPA Enzymatic assay for determining D-Ala/D-Asp, formation of NAD^+ which can be determined spectrofluorometrically
Model peptides			
[89]	RKKDVY RKDVY	Asp/Val	Gradient HPLC on YMC Basic B-03-5 with gradient 1.22% ACN min^{-1}, mobile phases 10 mM ammonium formate/1% MeOH/H_2O UV detection at 340 nm For MS detection (electrospray) mobile phases were 0.05% TFA/ACN/H_2O with gradient 1.5% ACN min^{-1} Analysis after derivatization with Marfey's reagent (Fig. 18)
[90]	Nal-CYWKVCT YA-Toc-EVVG F-Amp-FWKT	Various amino acids	GC on Chirasil-L-Val, derivatization with *N*-trifluoroacetamide and isobutylesters. Temperature gradient 65–220°C MS detection Analysis after acid hydrolysis (6 M HCl/110°C/24 h)
[91]	Various tripeptides	Ala/Val/Leu/Ile/Phe	Isocratic HPLC on Cosmosil 5 C_{18} with mobile phase different MeOH concentrations for the different peptides. UV detection at 254 nm
[92]	Various LDL and LLL tripeptides	Phe/Tyr/Trp	Isocratic HPLC on RP-C_{18} (specifications not described) with mobile phase 65% MeOH in H_2O UV detection at 219 nm Fluorescence detection λ_{ex} 280 nm and λ_{em} 347 nm

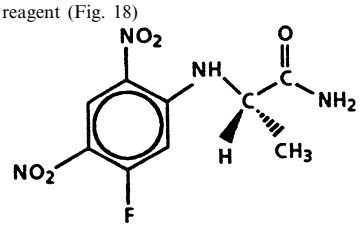


Fig. 18. Marfey's reagent.

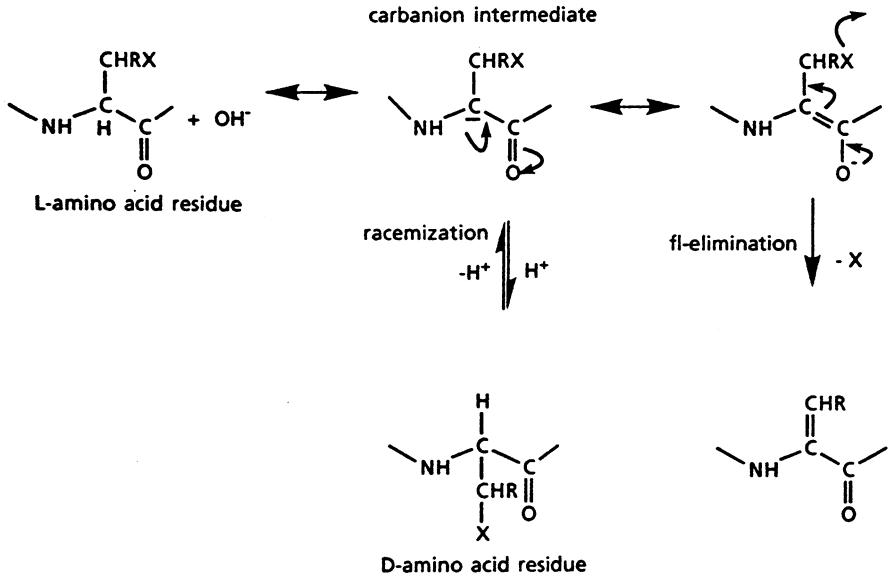


Fig. 14. Mechanism of racemization and β-elimination.

selection either in sample preparation or in the technique itself, except for optical rotation experiments. The latter, however, only indicates, whether the reaction took place or not. Racemisation does not necessarily mean that a racemic mixture is formed and that the optical rotation will decrease towards zero. If racemisation in single amino acids is studied with HPLC, derivatisation with a chiral reagent is necessary to create diastereomers. In case of racemisation studies on

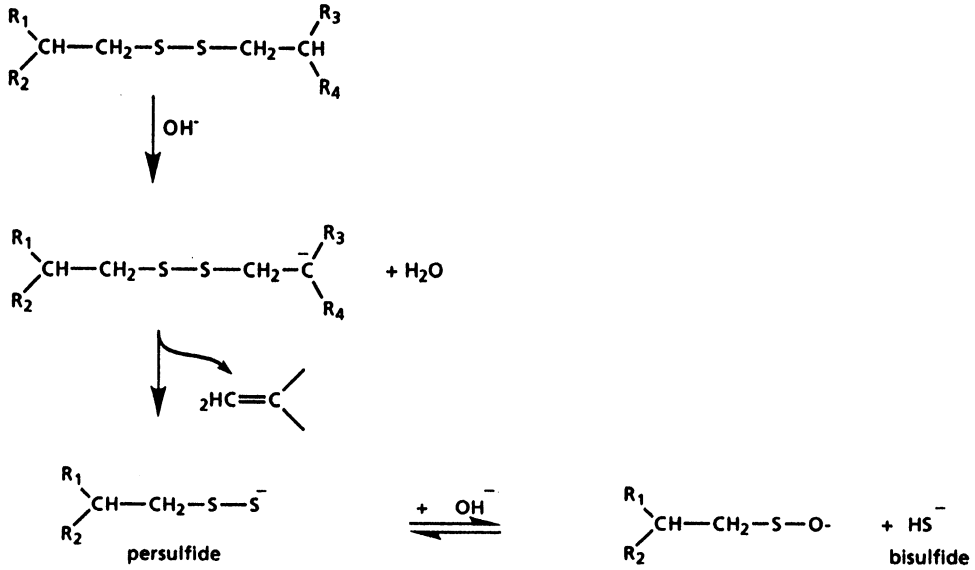


Fig. 15. β-Elimination of disulfides.

Table 7
Analytical techniques used to monitor β-elimination processes

Reference	Peptide/protein	Analysis
[93]	AFGP	Spectrophotometric analysis at 241 nm
[94]	Albumin	Cathode stripping voltammetry: mercury electrode, −0.35 V against saturated calomel electrode (deposition). Measurement of the HgS film was carried out by applying a −0.35– −1.0 V d.c. ramp at 83 mV s^{-1}. Peak measurement at −0.79 V
	α-Chymotrypsin A	Ion-selective electrode measurements
	Glutathion, insulin	Spectrofluorometric determination, measuring depression of the fluorescein mercury acetate fluorescence. λ_{ex} = 489 nm, λ_{em} = 520 nm
[95]	Insulin, lysozyme	5,5′-Dithiobis(2-nitrobenzoic acid) assay. Determination of free sulfhydryls. Molar extinction coefficient = 13 600 M^{-1} s^{-1}
[94]	Papain, pepsin	Cathode stripping voltammetry: mercury electrode, −0.35 V against saturated calomel electrode (deposition). Measurement of the HgS film was carried out by applying a −0.35– −1.0 V d.c. ramp at 83 mV s^{-1}. Peak measurement at −0.79 V Ion-selective electrode measurements Spectrofluorometric determination, measuring depression of the fluorescein mercury acetate fluorescence. λ_{ex} = 489 nm, λ_{em} = 520 nm
[96]	Phosvitin	Spectrophotometric analysis at 241 nm
[94]	Ribonuclease A	Cathode stripping voltammetry: mercury electrode, −0.35 V against saturated calomel electrode (deposition). Measurement of the HgS film was carried out by applying a −0.35– −1.0 V d.c. ramp at 83 mV s^{-1}. Peak measurement at −0.79 V Ion-selective electrode measurements Spectrofluorometric determination, measuring depression of the fluorescein mercury acetate fluorescence. λ_{ex} = 489 nm, λ_{em} = 520 nm
[95]	Ribonuclease A	5,5′-Dithiobis(2-nitrobenzoic acid) assay. Determination of free sulfhydryls. Molar extinction coefficient = 13 600 M^{-1} s^{-1}
[94]	Trypsinogen	Cathode stripping voltammetry: mercury electrode, −0.35 V against saturated calomel electrode (deposition). Measurement of the HgS film was carried out by applying a −0.35– −1.0 V d.c. ramp at 83 mV s^{-1}. Peak measurement at −0.79 V Ion-selective electrode measurements Spectrofluorometric determination, measuring depression of the fluorescein mercury acetate fluorescence. λ_{ex} = 489 nm, λ_{em} = 520 nm

the whole peptide or protein derivatisation is not a requisite, as diastereomers are formed during the reaction. Mass spectrometric analysis is only useful if the racemisation took place in e.g. a deuterated medium. In this way the site of racemisation can be determined after fragmentation spectra are recorded.

GC experiments are usually carried out with columns where L-Val is bound to the stationary phase. The method requires that peptides and proteins are hydrolysed and derivatised prior to analysis to determine the site of amino acid racemisation. No information can be obtained about the place of racemisation in the chain of the peptide and/or protein if the molecule contains more than one residue of the racemic amino acid.

The use of enzymatic analysis is very limited. Only the racemisation of Ala and Asp can be monitored with this assay [78].

8. β-Elimination

The β-elimination reaction mechanism is similar to that of racemisation (Fig. 14). The β-elimination of the SH group from the Cys residue in a protein resulting in the destruction of the disulfide bond is the cause of high-temperature and high pH-induced protein inactivation. The products, originating from this elimination reaction (Fig.

15) will contribute to physical instability conversions such as aggregation, adsorption and precipitation. Other amino acid residues that may undergo β-elimination reactions are Ser, Thr, Phe and Lys. The presence of metal ions has a catalysing influence on the reaction rate [2]. β-Elimination can be monitored by determining the product cleaved from the peptide or by monitoring the parent.

In Table 7, a summary of analytical techniques used to monitor β-elimination is given.

In the literature not much data are available about the analysis (both qualitative and quantitative) of β-elimination reactions. Very specific is the cathode stripping voltammetry method where the formation of the intermediate persulfide is monitored. β-Elimination in Cys-Cys leads often to products which can be compared to the products formed during reduction, meaning that quantifying the reaction is possible with similar techniques as used in reduction reaction studies. See Tables 1 and 6 for references to Figs. 16–18.

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