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

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Abstract

The physical instability of proteins and peptides as well as the various analytical techniques used to study the various aspects of physical instability have been reviewed. Physical instability of proteins and peptides involve changes in secondary, tertiary and quaternary structures of these compounds. After a general introduction of the subject the literature data of these changes and their analytical aspects have been summarized in a Table.

Keywords: Analytical techniques; Proteins; Peptides; Physical instability

1. General introduction

Instability of peptides and proteins can be divided into two forms: chemical and physical instability. This review concerns the analytical aspects of physical instability. Physical instability involves changes in the secondary, tertiary and quaternary structures of proteins. These changes can be caused by various factors such as temperature, pH, denaturating reagents, etc.

This concise review is divided in two parts: the first concerns the unfolding process of proteins and how to detect this process while the second part deals with aggregation and its qualitative and quantitative detection.

2. Unfolding of proteins and polypeptides and its detection

Proteins and polypeptides are, in contrast with oligopeptides, able to form secondary, tertiary and even quartenary structures. These 'superstructures' can undergo changes independent of any chemical modification.

An important physical change is the unfolding of the protein. This process results in the total disruption of the tertiary and frequently also the

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secondary structure [1]. A protein may unfold under a variety of conditions. Denaturing at high temperature, under the influence of salts or extreme pH results only in partly unfolding while high concentrations of guanidine HCl (GndHCl) and urea denaturate proteins into a totally unordered and unfolded state [1,2]. The exact physical explanation of the denaturating ability of urea and GndHCl is not clear. It is suggested, however, that, due to protein binding, both compounds increase the aqueous solubility of the hydrophobic portions of the proteins [3] and, moreover, urea denatures the protein by binding to the peptide bonds of the protein [4].

Unfolding of proteins can follow two pathways: a co-operative two state transition between the folded (F) and the unfolded state (U) (Eq. (1)) [1] or the transition of the folded to the unfolded state through an intermediate state (I) (Eq. (2)) [2]:

$$F \leftrightarrow U \quad (1)$$

$$F \leftrightarrow I \leftrightarrow U \quad (2)$$

Some intermediate states exhibit a high content of secondary and some tertiary structures and are called the molten globule state. This state is more compact than the unfolded form, however, the unique tight packing of side chains is absent and it exhibits no melting phenomenon [2].

Temperature- and urea- or GndHCl-induced unfolding of proteins are widely studied and a variety of analytical techniques are applied to follow the unfolding process of proteins.

In the case of temperature-induced unfolding the specific analytical technique differential scanning calorimetry (DSC) can be used because a protein unfolds by the uptake of energy. The process can be monitored easily with DSC whereby the difference in energy uptake (ΔH) between a reference and the protein is measured. Unfolding of a protein is an endothermic process with a maximum energy uptake at the transition, also called the melting temperature T_m, which is defined as the temperature where 50% of the molecules are unfolded. A higher T_m indicates a more physically stable protein [1,5–12].

The unfolding of proteins gives rise to the loss of secondary and tertiary structures resulting in changes in the spectroscopic properties. These changes can be exploited to study the protein unfolding. Alterations in the tertiary structure can be analysed by fluorescence and circular dichroism spectroscopy (CD) in the UV region. Unfolding of the tertiary structure leads to the exposure of Tyr and Trp to a hydrophilic environment, resulting in a red shift in the fluorescence spectrum (excitation and emission). The loss of the tertiary structure gives a decrease in Trp fluorescence [7,13,14]. Another approach is the use of the 1-anilino-8-naphthalenesulfonate (ANS) fluorometric assay. A protein is folded with the hydrophobic amino acids buried in the interior of the molecule [15]. The loss of the tertiary structure leads to exposure of these hydrophobic regions of the protein to the solvent. The fluorescent probe ANS has a strong affinity for the hydrophobic regions of the protein [16]. In aqueous solution, ANS itself shows only weak fluorescence. However, this increases in nonpolar environments like the hydrophobic surface of a protein with a blue shift in the emission maximum. ANS binds with preference to the molten globule state [17] of a protein and can therefore be used to detect whether the protein unfolds through a molten globule intermediate state or not.

With CD the difference in molar extinction coefficient of the R and L component in linearly polarised light (molar ellipticity, θ) is measured. Near UV CD measurements reveal the ellipticity of the aromatic groups fixed in specific orientations due to the tertiary structure. A decrease in ellipticity will be observed during unfolding [7,13,14,16–23]. The loss of the secondary structure can be followed by changes in the far UV CD spectra (200–250 nm) that originate from peptide groups in α-helices and β-sheets [19].

Techniques like NMR and Fourier Transform infrared (FT IR) spectroscopy are applied to characterise the secondary and tertiary structure of the folded, unfolded and intermediate states of proteins.

FT IR is often used to study the effects of temperature on the secondary structure. Here the investigated property is the C=O stretching vibrations of the peptide moiety, which are weakly coupled with the in-plane N–H bendings and the C–N stretching vibrations [24].

NMR analysis has been shown to be very useful in studying the structure of denaturated protein. One dimensional ^{1}H NMR, as well as two or three dimensional NMR (correlation of two or three spins, respectively), are applied for the characterisation of the folded, unfolded and intermediate states. The unique magnetic environments in the close-packed folded structure give distinct chemical shifts of the protons. Chemical shifts change when the protein unfolds, because contact shifts are lost when side chains are allowed to rotate freely [25–28].

The unfolding of the proteins leads to changes in the molecular shape and physical properties. These changes can be monitored with chromatographic and electrophoretic techniques. Folded proteins with a more compact structure have a larger elution volume than the unfolded proteins using size exclusion chromatography (SEC) [11,22,29], in contrast to electrophoresis where a larger molecule results in a slower migration velocity. An often used application in electrophoresis is two-dimensional electrophoresis. This technique appeared to be very helpfull in studying the effects of denaturants like urea on the migration behaviour of proteins.

The increase in volume of the protein upon unfolding is also studied by urea-gradient electrophoresis. Polyacrylamide gels with a linear gradient of urea perpendicular to the direction of electrophoretic migration are prepared. The protein is layered on top of the gel and migrates in continuously increasing concentrations of urea. At low urea concentrations the protein migrates with the mobility of the native protein and at high urea concentrations the protein migrates with the velocity of the unfolded protein. Transition of the protein to its unfolded state can be observed at intermediate urea concentrations [3].

By comparing these techniques it can be seen that techniques which can separate proteins on the basis of size and charge/mass ratio are SEC and the electrophoretic techniques, respectively. In SEC, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and capillary electrophoresis (CE) mechanistical information (Eqs. (1) and (2)) can be obtained. If the conversion rates are fast, however, SEC and SDS-PAGE are not useful because they have long analysis times. In CE the analysis time can be very short, thus enabling us to follow fast unfolding processes. From the techniques used to monitor the thermal induced unfolding all the methods except FT IR and NMR give indirect information of the process. Only in FT IR and NMR is information about the position of the amino acid side chains in the protein obtained. In studying temperature-induced unfolding the use of spectroscopic techniques like FT IR and NMR is also very appropriate because of the easy control of the temperature. However, spectroscopic techniques such as FT IR and NMR mostly yield data about the concentration of denaturating agents like urea and GndHCl needed to induce the loss of native conformation of the protein. Also the mechanism (Eqs. (1) and (2)) through which the unfolding proceeds can be determined (CD, fluorescence).

In Table 1 a summary of the analytical techniques used to monitor the unfolding of proteins and polypeptides is listed.

3. Aggregation

In the process of folding and unfolding partially unfolded intermediates exist. These intermediates may form large soluble or insoluble aggregates [4]. Hydrophobic interactions are probably the major driving forces of aggregation between (partly) denatured proteins [38].

To monitor aggregation various changes in the properties of the analytes can be used. The most important changes are the alteration in size and the formation of precipitates.

SEC and SDS-PAGE are useful for the analysis of soluble aggregates since these methods monitor the increase of protein size during aggregation. As mentioned above concerning the unfolding of proteins and polypeptides, the larger particles have a higher elution velocity with SEC and a lower migration velocity in SDS-PAGE. The distinction between covalently and non-covalently (ionic) bound aggregates can be made via non-reducing and reducing SDS-PAGE. Both reducing and non-reducing SDS-PAGE can be combined in two-dimensional electrophoresis. The first dimen-

Table 1
Analytical techniques used to monitor unfolding of proteins and polypeptides

Reference	Protein/peptide	Analysis
[14]	Apo-α-lactalbumin	Trp fluorescence at $\lambda_{ex} = 268$ nm or $\lambda_{ex} = 278$ nm
[19]	Bovine-α-lactalbumin	CD measurements, far UV in 0.1 cm cuvette, near UV in 1 cm cuvette ANS fluorescence at $\lambda_{ex} = 455$ nm
[30]	Bacteriorhodopsin	FTIR, temperatures 25, 66, 82, 92 and 97°C
[22]	Barstar	SEC on Superdex 75 HR 10/30 or Superose 6 HR 10/30 column, mobile phase 20 mM sodium phosphate, 0.2 M KCl, 1 mM EDTA, 1 mM DTT (pH 7.0) CD measurements, far UV in 0.1 cm cuvette, near UV in 1 cm cuvette Trp fluorescence at $\lambda_{ex} = 287$ nm, $\lambda_{em} = 332$ nm
[20]	β-Lactamase	CD measurements, no specifications Trp fluorescence at $\lambda_{ex} = 295$ nm PAG electrophoresis, horizontal urea gradient 1–8 M
[23]	Bromelain	CD measurements, far UV in 0.1 cm cuvette, near UV in 1 cm cuvette DSC, scanning rate 1°C min^{-1} over the range 40–80°C
[21]	Chaperonin GroEL	Gel filtration on Bio-Gel SEC 40-XL column, mobile phase 10 mM TRIS, 10 mM $Mg(CH_3COO)_2$, 200 mM KCl UV detection at 214 nm CD measurements, 0.1 cm cuvette for all measurements Trp- and ANS fluorescence at $\lambda_{ex} = 278$ and 485 nm, respectively
[26]	drk (N-terminal SH3 domain)	^1H NMR and ^{15}N NMR
[31]	GPA hexokinase	SDS-PAGE (not specified) under pressure Urea gradient electrophoresis on inverse polyacrylamide percentage gels (8–7%)
[32]	GRF	ESI-MS and MS/MS
[10]	Hevein	DSC, scanning rate 1°C min^{-1} over the range 20–120°C
[6]	hGH	DSC, scanning rate 10°C min^{-1} over the range 30–100°C
[33]	HtrA protease	FTIR, temperature scan between 25–95°C, steps of 5°C $scan^{-1}$ SDS-PAGE on 10% polyacrylamide
[32]	Melittin	ESI-MS and MS/MS
[12]	Firefly luciferase	FTIR, temperature scan between 4–70°C DSC, scanning rate 0.167°C min^{-1} over the range 4–70°C
[5]	Lysozyme	DSC, scanning rate 5°C min^{-1} over the range 30–100°C
[34]	Myofibrillar proteins	DSC, scanning rate 10°C min^{-1} over the range 10–100°C
[25]	Ribonuclease A	CD measurements, no specifications ^1H NMR
[24]	Ribonuclease A	FTIR, temperature scan between 20–73°C at a rate of 0.1°C min^{-1}, pressure scan between 0.1 and 1240 MPa
[27]	Ribonuclease A	^1H NMR, temperature scan between 7.5–40°C, pressure scan between 1 and 2000 atm
[14]	RNase (S54G/P55N variant)	Trp fluorescence at $\lambda_{ex} = 268$ or 278 nm
[7]	rp24	CD measurements, far UV in 0.01 cm cuvette, near UV in 0.1 cm cuvette Trp- and ANS fluorescence at $\lambda_{ex} = 295$ and 365 nm, respectively DSC, scanning at 1°C min^{-1} over the range 10–70°C
[11]	sCD4-PE40	SEC on Zorbax GF-250 column, mobile phase 0.1 M potassium phosphate, 0.9 M KCl, UV detection at 221 nm DSC, scanning rate ranged from 0.5 to 1°C min^{-1} over ranges varying from 20–90°C
[5]	Bovine somatotropin	DSC, scanning rate 5°C min^{-1} over the range 30–100°C
[35]	Transferrin	HPCE on 100 μm coated capillaries, running buffer 18 mM TRIS, 18 mM borate, 0.03 mM EDTA (pH 8.4), voltage 8 kV, UV detection at 280 nm

Table 1 (Continued)

Reference	Protein/peptide	Analysis
[28]	Trypsin inhibitor	^{1}H NMR, temperature scan between −2–+41°C
[16]	Tubelin	CD measurements, far UV in 0.1 cm cuvette, near UV in 1 cm cuvette Trp- and ANS fluorescence at $\lambda_{ex} = 280$ and 350 nm, respectively
[36]	Various Ala-based peptides	FTIR, temperatures 1, 25 and 50°C
[13]	Various proteins	FPLC on Bio-Sil-250 or Superose 12 column CD measurements, far UV in 0.1 or 0.02 cm cuvette, near UV in 1 cm cuvette Trp- and ANS fluorescence at $\lambda_{ex} = 280$ and 400 nm, respectively
[18]	Various proteins	CD measurements, 1 cm cuvette for all measurements
[29]	Various proteins	SEC on Superose-12 column, mobile phase 0.1 M sodium phosphate (pH 6.8) or 0.1 M sodium phosphate, 8 M urea, 0.01 M DTT (pH 6.8) or 0.1 M sodium phosphate, 6 M GndHCl, 0.01 M DTT (pH 6.8), UV detection at 226 nm CD measurements
[3]	Various proteins	Urea gradient electrophoresis on inverse polyacrylamide percentage gels (15–11%)
[37]	Whey proteins	ANS fluorescence at $\lambda_{ex} = 375$ nm DTNB assay to determine free sulfhydryls, UV detection at 412 nm and ε 13 600 M^{-1} cm^{-1} SDS-PAGE (non reducing) on 12.5% polyacrylamide

Table 2
Analytical methods used to monitor the aggregation of proteins

Reference	Protein	Method of analysis
[13]	Apomyoglobin β-lactamase	SEC on a Superose-12 column, mobile phases 0.5 M KCl (pH 7.0) for native proteins and 0.15–0.5 M KCl for aggregation studies, UV detection at 220 nm
[39]	Chaperonin GroEL–GroES complexes	SEC on Sepharyl S-300 column with mobile phase 2.5 mM AMP-PNP, 0.6 mM 6-His-*N*-DHFR, analysis after fractionation with SDS-PAGE (Coomassie Brilliant Blue staining) and scintillation spectroscopy STEM Equilibrium dialysis
[40]	Chaperonin GroEL–GroES complexes	SEC on Sepharyl S-300 column with mobile phase 20 mM MOPS, 10 mM KCl, 90 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT (pH 7.2), analysis after fractionation with SDS-PAGE SPR
[13]	Ccytochrome *c*	SEC on a Superose-12 column, mobile phases 0.5 M KCl (pH 7.0) for native proteins and 0.15–0.5 M KCl for aggregation studies, UV detection at 220 nm
[6]	hGH	SEC on a SEC Zorbax GF-250 column, mobile phase 50 mM ammoniumcarbonate with a flow rate of 0.6 ml min^{-1}, UV detection at 215 nm Turbidity measurement at 400 nm
[41]	Lens proteins	SEC on Superose-6 column, mobile phase 50 mM TRIS–HCl (pH 7.4), 2 mM EDTA, 0.14 M NaCl and 0.02% NaN_3 with a flow rate of 0.3 ml min^{-1}, UV detection at 280 nm
[42]	Duck salt-soluble proteins	Turbidity measurements at different temperatures at 320 nm SDS-PAGE on 8–16% polyacrylamide linear gradient, Coomassie Brilliant Blue R-250 staining
[43]	Glutenin protein	Reducing and non-reducing SDS-PAGE on 4, 6, 8, 10, 12 and 14% polyacrylamide, Coomassie Brilliant Blue staining
[44]	Soy protein isolate components	Reducing and non reducing SDS-PAGE on 5–15% polyacrylamide, Coomassie Brilliant Blue R-250 staining Two dimensional SDS-PAGE with 5–15% polyacrylamide in the first (non-reducing) and second (reducing) dimension, densitometric detection at 570 and 395 nm
[13]	Staph. nuclease	SEC on a Superose-12 column, mobile phases 0.5 M KCl (pH 7.0) for native proteins and 0.15–0.5 M KCl for aggregation studies, UV detection at 220 nm

sion is a non-reducing run and the second dimension a reducing one.

Other techniques described to monitor the aggregation are turbidity measurements, scanning transmission electron microscopy (STEM), equilibrium dialysis and surface plasmon resonance (SPR).

Aggregation is well characterised by the methods that discriminate on the basis of size. SPR gives information about the aggregation velocity, enabling the investigation of the kinetics of aggregation. Sizes sometimes become so large that visualisation with STEM and turbidity measurements is possible. With turbidity measurements the kinetics of the aggregation can be monitored.

In Table 2 an overview of the analytical techniques used to monitor the aggregation of proteins and polypeptides is given.

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