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Protein homogeneity testing by reversed-phase high-performance liquid chromatography of reduced and non-reduced samples

RAPID COMMUNICATION

Summary — The concept of this study was based on the assumption that protein standards obtained using the same method may reveal heterogeneity explainable by changes in the disulphide bonding pattern. Two lots of bovine α-lactalbumin standard were subjected to reversed-phase high-performance liquid chromatography (RP-HPLC). One of them was homogenous. The other revealed the presence of two major fractions when non reduced. Neither RP-HPLC after protein reduction nor mass spectrometry showed differences between them. This indicates that one of the lots contained isomers with different locations of disulphide bonds. A comparison of RP-HPLC profiles obtained with and without reduction may be thus recommended as a tool for testing homogeneity of protein standards, especially those used in experiments involving denaturation.
Key words: disulphide bonds, α-lactalbumin, protein standard heterogeneity, reversed-phase high-performance liquid chromatography.

Disulphide bonding pattern is one of crucial factors determining protein properties [1, 2]. Changes in the disulphide bonding pattern may affect *e.g.* protein susceptibility to further aggregation [3, 4]. An example of the influence of the disulphide bonding pattern on the biological activity of peptides is given in ref. [5]. The location of disulphide bonds in the protein chain may be affected *e.g.* by heating. This process causes both changes in the intrachain disulphide bond location in protein monomers and aggregation *via* interchain disulphide bond formation. The reaction rate may be enhanced in the presence of a donor of free sulfhydryl groups [6, 7].

The purity of protein standards used in such experiments is usually checked using gel electrophoresis after reduction or, more recently, by mass spectrometry. Gel electrophoresis may detect contamination of the standard by other proteins. Simple molecular weight measurement may also detect chemical or enzymatic modifications, as well as amino acid insertions, deletions or substitutions. However, none of these methods can detect changes in disulphide bonding patterns, if they do not lead to aggregation. Isomers with different disulphide bonding patterns (so called "scrambled isomers") may be identified using proteolysis followed by identification of the resulting peptides by mass spectrometry [6, 7].

Bovine α-lactalbumin (α-LA) (Accession No P00711 in the SWISS-PROT database [8]) is recommended as a model protein in experiments aimed to study protein folding/unfolding phenomena [9]. α-LA belongs to the major bovine milk proteins [10]. It forms scrambled isomers which may be easily separated using reversed-phase high-performance liquid chromatography (RP-HPLC) [6, 7]. During heating of milk α-LA can interact with β-lactoglobulin, a protein containing free sulfhydryl groups [11].

In the present study we intended to show, using α-LA as an example, that protein standards reveal heterogeneity which may be explained as an effect of disulphide scrambling. Such heterogeneity may be detected by comparing the results of RP-HPLC with and without reduction.

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EXPERIMENTAL

Materials

Two lots of bovine α-LA (Cat. no. L6010) isolated according to ref. [12] were purchased from Sigma.

The acetonitrile (ACN), trifluoroacetic acid (TFA) (HPLC grade), 2-mercaptoethanol and 1,3-bis[tris(hydroxymethyl)-methylamine]propane (Bis-Tris) (ultrapure bioreagents) were purchased from Baker.

Sinapinic acid used as a matrix for mass spectrometric measurement was purchased from Amersham Biosciences.

Other reagents (analytical grade) were purchased from POCh.

Water was deionized using MilliQsystem (Millipore).

Methods

The chromatographical analysis was carried out using Shimadzu assembly consisting of two LC-10AD pumps, SIL-10AD autosampler, SCL-10AD controller, CTO-10AS column oven and SPD-M10AW PDA detector. TSK-Gel® C_{18} NPR column 35×4.6 mm (Tosoh Biosep) was used. Class-VP 5.03 software (Shimadzu) was applied for data acquisition.

Solvents containing ACN, H_2O and TFA (100:900:1 and 900:100:0.7 v/v/v for solvents A and B respectively) [13] were used for HPLC separations. Protein samples were dissolved in 0.05 M phosphate buffer (pH = 6.6) to concentration *ca.* 2 mg · mL^{-1}. 400 μL of resulting solvent was then mixed with 600 μL of solvent A containing 6 M urea with pH = 2.2 adjusted using TFA [13]. The procedure of reduction was adapted from ref. [12]. 20 μL of 2-mercaptoethanol was added to 400 μL of protein solution (2 mg · mL^{-1}) in 0.1 M Bis-Tris/HCL buffer with pH = 6.6. Reduction time was 1h at room temperature. The resulting solvents were then mixed with 600 μL of solvent A containing 6 M urea with pH = 2.2.

The following gradient was used: 25 % of solvent B for a start, 33 % B after 1 min, 35.2 % B after 4 min, 35.2 % B after 10 min, 50 % B after 13 min, 60 % B after 16 min. After completion the gradient column was washed and equilibrated as follows: 95 % of solvent B after 16.5 min, 95 % B after 18.5 min, 25 % B after 19 min, 25 % B after 23 min. The following values of other parameters were applied: data acquisition time was 20 min, flow rate 0.6 mL · min^{-1}, temperature 30 °C and injection volume 10 μL. The fractions of α-LA were collected. Acetonitrile was evaporated in vacuum at 30 °C. Samples were then frozen and lyophilized.

Molecular weights of the fractions were measured using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences) with Ettan MALDI Control and Ettan MALDI Evaluation software (Amersham Biosciences).

Samples were prepared using the "dried droplet" technique [14]. The protein lyophilizates were dissolved in 10 μL of solvent containing ACN, H_2O and TFA (50:50:0.5 v/v/v). The resulting solutions were mixed with the solution of sinapinic acid (10 mg · mL^{-1}) in the same solvent (1:2 v/v). 0.2 μL of mixture was loaded onto the slide. Crystallization occurred at room temperature.

Mass measurements were performed in the linear mode with extraction of positive ions at an acceleration voltage of 20 kV. 200 laser pulses were used for one spectrum acquisition. α-lactoglobulin not subjected to HPLC was used for external calibration. Its molecular weight was checked using MALDI-TOF and quadrupole-time-of-flight (Q-TOF) mass spectrometry, independently in two laboratories.

RESULTS AND DISCUSSION

Chromatograms of two lots of bovine α-LA are presented in Fig. 1. The lot whose chromatogram is shown in Fig. 1a formed a major peak and minor components with different retention times. The other lot formed two major peaks (Fig. 1b). After reduction both lots formed homogeneous peaks with the same retention times (Fig. 1c and d).

MALDI-TOF spectra of the first lot (Fig. 2a) and two major fractions of the other one (Fig. 2b and 2c) did not reveal detectable differences between particular monomers of α-LA. Differences in molecular weights can be considered as uncertainty. The equipment and proce-

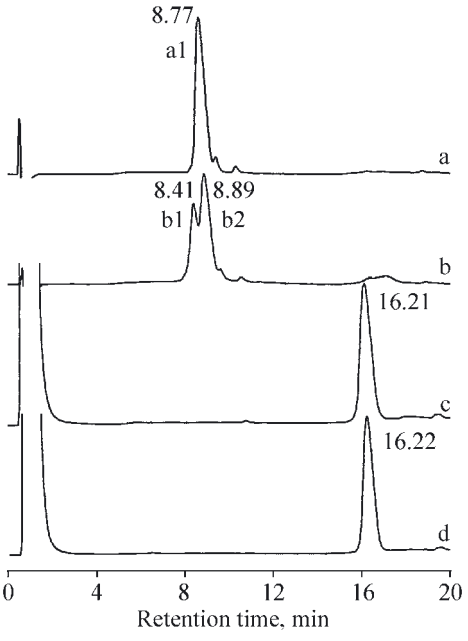

Fig. 1. Chromatograms of bovine α-lactalbumin: a) lot 1 without reduction, b) lot 2 without reduction, c) lot 1 after reduction, d) lot 2 after reduction; a1, b1 and b2 — fractions whose MALDI-TOF-MS spectra are presented in Fig. 2

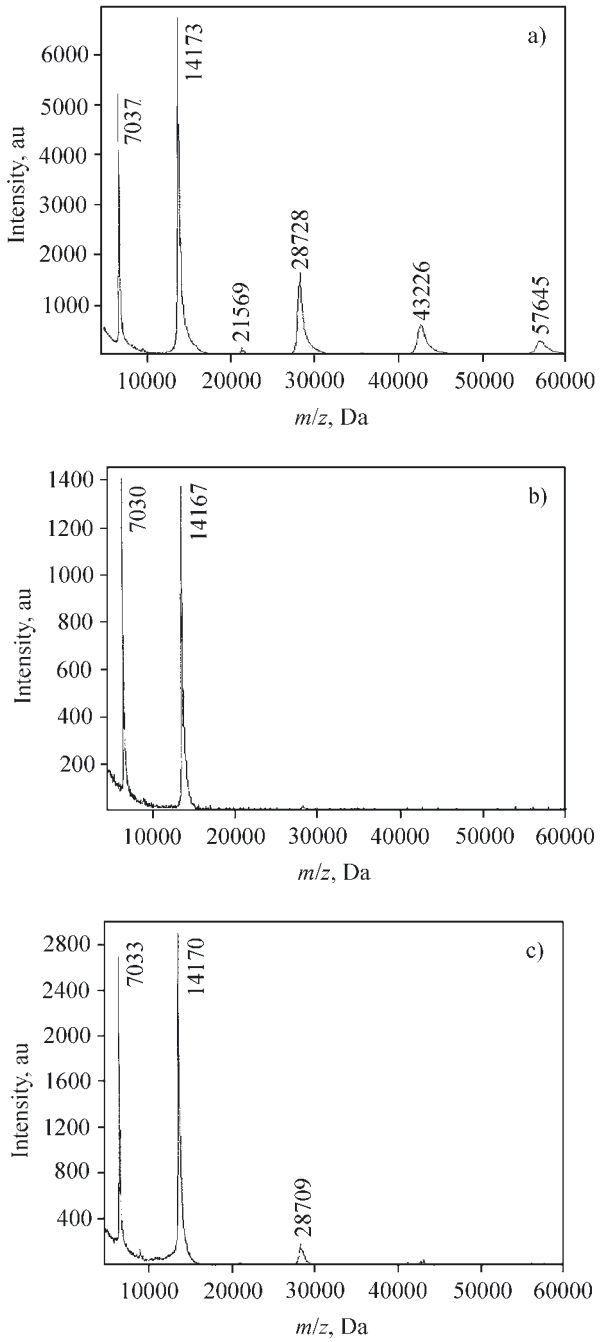

Fig. 2. MALDI-TOF-MS spectra of non-reduced bovine α-lactalbumin fractions: a) lot 1 (fraction a1 in Fig. 1), b) lot 2 (fraction b1 in Fig. 1), c) lot 2 (fraction b2 in Fig. 1)

dure used enables to measure molecular weights of proteins with an error up to 0.2 % [15]. Peaks of weights to charge ratio higher than 14 173 Da (calculated weight of non-reduced α-LA with 1 H^+ cation is 14 179 Da) may be identified as the products of protein aggregation occurring during measurement [15].

Identical retention times after reduction and molecular weights allow to exclude heterogeneity caused by changes in the amino acid sequence [13] or glycosylation [16] affecting both the retention time of α-LA after reduction and molecular weight. Methionine oxidation could be the third possible reason of heterogeneity. This modification cannot give a detectable shift in retention time in the case of α-LA [17]. There is only one possible explanation of the differences in the chromatographic patterns of two protein lots. Loss of heterogeneity due to reduction suggests that one of them contained at least two scrambled isomers. The most likely explanation of this fact is that protein had undergone mild heating [11]. Although the isolation procedure [12] does not include heating, the heterogeneous α-LA could be isolated from heated milk.

The formation of scrambled isomers is not a specific property of α-LA. Scrambled isomers separable by RP-HPLC are also formed by other proteins, such as hirudin [18], ribonuclease A [19], α-thrombin [20] and lysozyme [21]. Thus, RP-HPLC with and without reduction seems to be an efficient tool for testing the homogeneity of proteins forming scrambled isomers or aggregates due to changes in the disulphide bonding pattern. Such a test may be applied provided that the reduction of protein causes significant changes in its retention time.

CONCLUSION

RP-HPLC with and without reduction can be recommended as a quick and simple method to control the protein isolation procedure or standard homogeneity in the case when it is important to obtain a protein characterized by a given disulphide bonding pattern.

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W kolejnym zeszycie ukażą się m.in. następujące artykuły:

— Sole *sec*-butylotrifenyloboranowe barwników hemicyjanowych jako efektywne inicjatory polimeryzacji rodnikowej inicjowanej fotoindukowanym przeniesieniem elektronu. Cz. II. Badania kinetyczne i zastosowanie teorii przeniesienia elektronu (*j. ang*.)

— Właściwości strukturalne bioaktywnych fragmentów uwalnianych z wybranych białek zwierzęcych w wyniku ich proteolizy (*j. ang*.)

— Wpływ jednostkowej energii wyładowań koronowych na zwilżalność poliolefinowej folii napełnionej

— Ocena skuteczności wzmacniania polietylenów mączką drzewną

— Żelowanie poli(chlorku winylu) w procesie walcowania

— Montmorylonit wyodrębniany z bentonitu — modyfikacja i możliwość wykorzystania w polimeryzacji anionowej ε-kaprolaktamu do otrzymywania nanokompozytów

— Ocena poprawki Bagleya na podstawie pomiarów w linii wytłaczarskiej

— Wpływ kwasu butanotetrakarboksylowego i chitozanu na właściwości bezformaldehydowej apretury tkanin bawełnianych