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Structural modifications of globular proteins in an ultrafiltration loop as evidenced by intrinsic fluorescence and reverse-phase liquid chromatography

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ABSTRACT

Occurrence of denaturation of proteins during ultrafiltration (UF) was already discussed in literature but it must be underlined that no general trends were drawn explaining the versatile observed behaviours. This paper aims at proposing a complementary approach focused on slight denaturation of proteins due to mechanical stress in a UF loop. This methodology has required powerful analytical tools as it aims at revealing slight denaturations leading to proteins remaining in soluble states and not to precipitation that is quite easily shown. Intrinsic fluorescence and Reverse Phase HPLC (RP-HPLC) techniques were selected to highlight subtle changes in protein structure. Mainly, only highly enriched fractions of proteins were available (with a more than 95% purity), but it is a limitation to the use of intrinsic fluorescence that is very sensitive to occurrence of minor protein contaminants. Then RP-HPLC was used not only for quantification of remaining soluble proteins but also to show slight changes in the protein peak shape, only evidenced with elution gradients specially established for this study to reach the goal. The main novelties of this paper deal with: (i) the use of a wide variety of globular proteins. A set of five proteins with different structural characteristics (molecular weight, overall hydrophobicity, occurrence of quaternary structures or not) was used for the demonstration, and (ii) the selection of specific RP-HPLC elution conditions able to highlight protein structural evolution with respect to UF requirement, especially concerning the use of low concentrations (1 g L^{-1}) to avoid denaturation due to highly concentrate media. An in-depth discussion based on protein peak shape was proposed and (iii) the establishment of model experiments that voluntary led to denatured proteins only obtained by mechanical stress at 45 °C, conceptually different than denaturation obtained by external physico-chemical variations such as addition of chemical compounds modifying protein structure as commonly used to denature proteins for analytical purpose. These model experiments were based on establishment of various shear stresses obtained by more or less quick stirring from zero to several thousand rotations per minute allowing establishing a reference scale of protein denaturation states with a Reynolds number varying in a wide range.

Cross-flow UF of each single protein solution was performed in batch mode during 5–6 h at 45 °C, retentates were analysed and compared to the reference scale of mechanical denaturation obtained from the model experiments.

As a general conclusion, it seemed that proteins can be classified according to a "soft/hard" criterion depending on the protein own ability to be denatured by contact with a surface. The "soft" proteins were partly denatured by the combination of shear stress and temperature in model experiments and in UF, whereas the "hard" proteins of quite stable structure were not modified. The behaviour during UF was sometimes much more complex, especially when the protein were capable of crossing the membrane.

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1. Introduction

For many years, the industrial fractionation of milk components has been mainly carried out by using chromatography and membrane technologies [1].

Among these separation techniques, ion exchange chromatography (IEC) is widely used and leads to highly enriched protein fractions. In IEC the extraction is commonly based on two main steps: a first retention step on the chromatographic support and an elution step using a variation of the eluent ionic strength or of its pH [2]. This chromatographic process is considered as a non-denaturing reference operation. However, in the case of metalloproteins, such as α-lactalbumin (Ca) and lactoferrin (Fe), IEC displaces the inorganic cations from a specific structural region of the native proteins (holo form). Then addition of a similar cation issued from a quite concentrated inorganic salt solution is performed on eluted protein (without cation, apo form) to obtain the end form containing the cation. But no proof of the exact location of the up-taken cation has been evidenced and it is not certain that the final protein corresponds to the holo form of the native structure.

Ultrafiltration (UF) is probably the best candidate for an alternative process to chromatography with no need of de-chelation and re-chelation of the cation absolutely required by IEC and opening the discussion about the protein structural integrity. Regardless of the mode of operation (single or cascade), UF usually leads to target protein enriched fractions, generally less pure than those obtained by IEC, but valuable in complementary formulations [3–5]. Membrane processes are generally considered as "soft" processes inducing little or negligible negative impact on biological fragile molecules. This belief dates back to 1980–1990s and was mainly funded on occurrence, or non-occurrence of protein precipitation in the filtration loop. Precipitation mainly results from non-quantified mechanical forces occurring both in the UF loop (circuit, valves, pumps, etc.) and in the membrane material itself during permeation [6]. However, with time, perception by process specialists of the exact meaning of protein denaturation has evolved and it is now well understood that structural modifications of proteins can either lead to precipitation or to soluble states. This evolution is mainly due to the growing accessibility of more and more sophisticated and powerful analytical tools.

Finally, all soluble forms of a protein that are different from the native state can be called "denatured states"; but the denaturation did not systematically induced the loss of target functionalities, such as technological function as foaming and so on. Nevertheless there is not one single denatured state but several ones and the exact description of these states remains sharply dependent on the chosen analytical methods. In this context, and due to the growing interest in protein fractions of high added value, structural evolution of soluble proteins submitted to UF must be re-investigated with modern and appropriate analytical techniques, such as HPLC and intrinsic fluorescence.

The aim of this paper is to re-evaluate the structural evolution of various soluble proteins occurring in a UF loop. In order to focus on the impact of mechanical stress induced by the circulation in the filtration loop, the UF membrane cut-off was chosen in order to have high protein retentions; generally greater than 90%. Modifications of the proteins were followed using intrinsic fluorescence and RP-HPLC [7–12].

The main novelty of this paper deals with:

- the use of a large variety of globular proteins. Five globular proteins of various characteristics (molecular weight, isoelectric pH, hydrophobicity, ability to form aggregates) were used,
- the selection of Reverse Phase HPLC (RP-HPLC) experiments achieve in a non-classical manner and with an in-depth discussion of denaturation revealed by peak shape evolution. Moreover the paper underlines how the intrinsic fluorescence used as single analytical tool was not always sufficient to conclude on protein structural evolution in a valuable way,
- the establishment of model experiments that voluntary led to denatured proteins by mechanical stress further compared to states obtained in UF experiments.

2. Experimental

2.1. Proteins

A set of five globular proteins of various structural properties has been selected. Characteristics of these proteins, Bovin Serum Albumin (BSA), β-lactoglobulin (β-LG), lactoferrin (LF), lactoperoxidase (LP) and lysozyme (Lys) are given in Table 1. All these proteins are issued from ion exchange chromatographic processes, explaining thus the pH always lower or greater than their isoelectric pH (pHi). The aqueous solutions of proteins are prepared at 1 g L^{-1} without adjustment of pH.

- *Bovine serum albumin (BSA)* (Sigma A-2153-Lot 67 H0359, purity: 96%, Sigma Aldrich CHEMIE Steinheim, Germany) (natural pH = 6.4).

In its native form, BSA is known to be a mixture of both monomer and dimer, with a dimer to monomer ratio of about 5/95. BSA owns two tryptophan residues: Trp 212 is located in a hydrophobic binding specific site whereas Trp 134 is located at the molecule surface [13–17]. This protein was selected because its easily obtainable structural change and thus it can be used as a reference for the validation of the overall approach (we were *a priori* sure that structural evolution would be obtained by thermal treatment or gentle stirring, that was confirmed by results – see below).

Table 1
Characteristics of proteins [14,21,22,47].

Proteins	Molecular weight (g mol^{-1})	pHi	Number of S–S (SH)	T_D (°C)[a]	Number of Trp/monomer	R_S (nm)[b]	Hydropathicity (GRAVY)[c]
BSA	66,267 (monomer)	4.7–5.1	17 (1 SH)	62.2	2	3.5	−0.458
β-LG variants A and B	18,000 (monomer) 36,000 (dimer)	5.2–5.4	5 (1 SH)	72.8	2	2.0 (mono) 2.6 (dimer) 4.4 (octamer)	−0.01
LF	77,000–80,000	8.6–9.2	17	65 (apo) 90 (holo)	9	2.2 (mono) 4.4 (tetramer)	−0.289
LP	77,000–80,000	8.5–10.2	6 (3 SH)	nd	13	nd	
Lys	14,300	11	4	nd	6	1.8	−0.542

[a] T_D: Temperature of thermal denaturation.
[b] Stokes radius, nd: not determined.
[c] GRAVY: Grand average of hydropathicity index indicates the solubility of the proteins: positive GRAVY (hydrophobic), negative GRAVY (hydrophilic).