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Heat-induced denaturation/aggregation of porcine plasma and its fractions studied by FTIR spectroscopy

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ABSTRACT

The aim of the present work is the in depth study of the protein aggregation mechanisms of whole porcine plasma and its fractions (serum, albumin and globulins) during heating using FTIR spectroscopy. Also, 2D correlation spectroscopy (2D COS) was used to establish the sequence of events during heat-induced gelation for all fractions. The results indicate that serum albumin quickly aggregates from 70 °C through non-native intramolecular β-sheets while globulins show lower susceptibility to protein aggregation. When found together, the aggregation pattern strongly depends on the composition of the protein mixture. That makes the great difference between plasma (serum albumin + globulins + fibrinogen) and serum (serum albumin + globulins) behavior, with the aggregation degree at the end of the thermal process being enhanced in the presence of fibrinogen – and achieving a similar level to that of serum albumin – while minimized in its absence. Attending on the low content of fibrinogen in plasma, our results suggest a great fibrinogen ability to alter the thermal serum albumin and globulins behavior by modifying the negative interactions established between them when no more proteins are found in the media. Moreover, it is noteworthy the slow plasma aggregation pattern at the beginning of the thermal process relative to serum albumin, this way allowing a higher protein unfolding. This could be related to the high heat-induced gel properties of plasma. Also, 2D COS indicates that the sequence of events is very similar for the all analyzed samples, with α-helix being more thermo-labile than native β-sheet structure.

1. Introduction

Mammalian blood plasma is a complex straw-colored fluid with relatively high protein content (6–7%) that, as a whole, shows good techno-functional properties, especially in reference to its ability to form heat-induced gels (Dàvila, Parés, Cuvelier, & Relkin, 2007a; Hermansson, 1982; Hickson, Dill, Morgan, Suter, & Carpenter, 1980; Howell & Lawrie, 1984; Parés, Saguer, Saurina, Suñol, & Carretero, 1998; Saguer, Fort, Alvarez, Sedman, & Ismail, 2008). Although plasma actually is a complex mixture of over 3000 proteins (Garbett, Miller, Jenson, & Chaires, 2007); serum albumin (50–60%), globulins (36%) and fibrinogen (3–4%) are the most abundant (Putnam, 1975) and govern the overall plasma functionality. Each of the main protein fractions exhibits a particular functionality profile as a result of the protein molecular structure and the extent of protein–protein interactions under varying physicochemical conditions (Damodaran, 1996; Dàvila, Parés, Cuvelier, et al., 2007a; Dàvila, Saguer, Toldrà, Carretero, & Parés, 2007c; Kilara, 1984; Kinsella, 1976; Matsudomi, Oshitaa, & Kobayash, 1994). For example, the major component, the serum albumin, is a globular protein of molecular weight ~66 kDa, with a pI ~4.8 – which shifts to ~5.3 when defatted (Peters, 1985; Tanford, Swanson, & Shore, 1955) – has been considered the main contributor to heat-induced plasma gel formation (Fretheim & Gumpen, 1978; Harper, Suter, Dill, & Jones, 1978; Lee & Hirose, 1989). The propensity of serum albumin to gelation is attributed to the high number of disulfide bonds (17) in the molecule and the presence of a highly flexible free –SH-group (Cys^{34}) with a relatively low pK_a value (5–7) compared to that of free cysteine and glutathione or even of cysteines found in other proteins (8–11) (Carter & Ho, 1994; Owusu-Apenten, Chee, & Hwee, 2003; Pederson & Jacobsen, 1980; Peters, 1996; Shaked, Szajewski, & Whitesides, 1980; Svenson & Carlsson, 1975). The Cys^{34} is located within the protein and becomes exposed to solvent upon thermally-induced unfolding of the protein. This promotes the intermolecular SH/SS interchange reactions which contribute to gel formation (Catsimpoolas & Meyer, 1970; Lee & Hirose, 1989; Legowo, Imade, Yasuda, Okazaki, & Hayakawa, 1996; Opstvedt, Miller, Hardy, &

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Spinelli, 1984; Shimada & Cheftel, 1988; Yasuda, Nakamura, & Hayakawa, 1986). However, serum albumin cannot form gels as strong as those formed by whole plasma (Dàvila, Parés, Cuvelier, et al., 2007a), suggesting that synergistic interactions between different plasma proteins contribute to the observed properties of heat-induced plasma gels (Dàvila, Parés, & Howell, 2006; Dàvila, Parés, Cuvelier, et al., 2007a; Dàvila, Parés, & Howell, 2007b; Howell & Lawrie, 1984). For example, specific interactions between serum albumin and fibrinogen involving intermolecular disulfide bond formation and hydrophobic interactions can result in stronger heat-induced plasma gels relative to those obtained from the serum fraction – obtained after removing fibrinogen from plasma (Dàvila, Parés, Cuvelier, et al., 2007a; Dàvila, Parés, & Howell, 2007b). Also, heat-induced serum gels show better textural properties than gels obtained from globulins alone or, especially, serum albumin alone, also suggesting a synergistic effect when they are present together (Dàvila, Parés, Cuvelier, et al., 2007a). Immunoglobulins, the most abundant proteins in the globulin fraction (~50%), display a symmetrical Y-shaped structure composed of 2 heavy chains (~50 kDa) and 2 light chains (~25 kDa), held together by both disulfide bonds and non-covalent interactions but showing differences in types and/or number of interactions between molecules. Moreover, intrachain disulfide bonds are also present (Coico & Sunhine, 2009, p. 391). IgG pI value ranges from ~4.5 to ~10 because of their particular polyclonal origin and the existence of several isotypes, but with the most abundant ones showing values in the basic pl region – from ~7 to ~9.5 – (Jin, Luo, Oka, & Manabe, 2002; Prin, Bene, Gobert, Montagne, & Faure, 1995). Moreover, IgGs also possess multiple fibrinogen binding sites (Boehm & DeNardin, 2008).

The importance of fibrinogen in plasma gelation can be related to its own molecular characteristics, which can favor intermolecular interactions (Shimada & Matsushita, 1980; Wang & Damodaran, 1990; Wang & Lin, 1994). Fibrinogen is a fibrous glycoprotein of high-molecular weight (~340 kDa) with a complicated molecular structure consisting of two identical subunits. Each one is composed of three non-identical polypeptide chains (Aα, Bβ and γ), held together by 29 disulfide bonds. Its conformation is organized in a central globular hydrophobic nodule (E domain) containing the N-terminals of all six chains and two peripheral globular hydrophobic nodules (D domains) (Brown, Litvinov, Discher, & Weisel, 2007; Colafranceschi, Giuliani, & Colosimo, 2008; Doolittle, Goldbaum, & Doolittle, 1978) that consist of the C-terminal ends except of that corresponding to Aα chains (αC regions), which interact intramolecularly with each other at the central E region through their hydrophobic regions (Litvinov et al., 2007; Medved, Gorkun, & Privalov, 1983; Tsurupa, Tsonev, & Medved, 2002; Weisel & Medved, 2001). However, under specific conditions, αC domains can dissociate and become available for intermolecular interactions. Fibrinogen shows a negative net electrical charge at physiological pH, due to the abundance of Asp and Glu residues (Triantaphyllopoulos & Triantaphyllopoulus, 1967), with its pI being close to pH 5.5; there is a certain variability in this value because of the structural heterogeneity of fibrinogen, particularly the γ chain, which coexists in two different isoforms that differ from each other in charge but not in size (Henschen-Edman, 1995; Mosesson, Finlayson, & Umfleet, 1972; Mosher & Blout, 1973; Wasilewska, Adamczyk, & Jachimska, 2009). This can favor its interaction with basic IgG. Furthermore, protein–protein interactions due to the presence of fibrinogen may entail some protective effect of α-helices during heating, minimizing the formation of intermolecular β-sheet structure (Dàvila, Parés, & Howell, 2007b).

The correlation between the rheological properties of gels and the rate of aggregation through formation of intermolecular β-sheets during heating has been demonstrated in our laboratories. In the present work, we focus on studying the effect of changes in the secondary structure of plasma fractions (serum, serum albumin and globulins) on the thermally-induced gelation mechanism of whole plasma. The examination of the role of each protein fraction and combinations thereof on the changes in the protein unfolding pathway and extent of intermolecular β-sheet formation has been undertaken to gain a better understanding of the mechanism of gelation. For this purpose, Fourier self-deconvolution (FSD) of the amide I′ region in the FTIR spectra – strongly dominated by the C=O stretch vibration, which is highly sensitive to the secondary structure – is employed to monitor changes in secondary structures upon heating while the *generalized 2D correlation spectroscopic analysis* (2D COS) technique is applied to analyze the sequence of events leading to heat-induced protein unfolding and intermolecular association for whole plasma and its fractions.

2. Materials and methods

Porcine blood (4 L) was collected in an industrial abattoir using a sterile container, which contained sodium citrate solution (1% w/v final concentration) as anticoagulant. Blood was immediately chilled and kept at low temperature until processed. At the laboratory, the blood sample was centrifuged at 2530×*g* and 5 °C for 15 min (SORVALL RC 5C Plus, Dupont, Newtown, USA) to separate the plasma from the cellular fraction. From the obtained plasma, an aliquot (200 mL) was dialyzed until electrical conductivity values of ~4 mS cm^{-1} were reached and subsequently freeze-dried. The remaining plasma (~900 mL) was used to obtain different plasma protein fractions (see Plasma protein fractionation section). The moisture (ISO R-1442), protein (ISO R-937, conversion factor for nitrogen: 6.25) and ash (ISO R-936) contents of the freeze-dried material were 5.4%, 94.0% and ~0.5%, respectively.

2.1. Plasma protein fractionation

Salting-out and dialysis were sequentially combined to obtain three different plasma protein fractions (blood serum, globulins and serum albumin) from fresh plasma. The first step was carried out using a saturated ammonium sulfate solution prepared in 10 mM Tris/EDTA pH 7.4 at 4 °C as precipitating agent, which was slowly added until the desired salt saturation percentage was reached. At 20% saturation, the fibrinogen was precipitated, removed by centrifugation (10 000×*g* at 4 °C for 15 min), and discarded. An aliquot (200 mL) of the supernatant was kept as the blood serum fraction; and the remainder was adjusted to 60% saturation with the ammonium sulfate solution to precipitate the globulin fraction, which was recovered by centrifugation under the same conditions as given above for the separation of fibrinogen. The saturated ammonium sulfate solution was added to the resulting supernatant to reach 70% saturation in order to precipitate the serum albumin fraction, which constituted the pellet remaining after centrifugation. The latter two protein precipitates were washed once and finally re-dissolved in 10 mM Tris/EDTA pH 7.4. Precipitating salts were removed by exhaustive dialysis of the three plasma fractions against Milli-Q water at 4 °C using a membrane of 12–14 kDa pore diameter (Medicell International Ltd., London, UK) until electrical conductivity values of ~4 mS cm^{-1} were achieved. After dialysis, solutions were immediately frozen at −80 °C and freeze-dried in a VIRTIS Unitop SQ (Virtis, Gardiner, NY, USA) at −15 and 15 °C for the primary and secondary drying stages, respectively. In all cases, the water and protein contents of the obtained powders, determined as indicated above, were 5–7% and 93–94%, respectively.